The Antiviral Role of Cytokines.

By

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Statement.

All the work presented in this thesis was performed by me except for the following;

Reactive nitrogen intermediate (RNI) assays were performed by Melissa Awburn;

IL 1 assays were done by Sarah Osvath;

The univalent Fab' fragments of Mab 32 were prepared by Louise Furphy.

The L-arginine inhibitors and two of the cyclooxygenase/lipoxygenase inhibitors were made in Dr. Bill Cowden's laboratory.

The work described has not been submitted previously for a degree at this or any other university.

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Abstract.

During the 1980s much progress was made in elucidating antiviral properties and mechanisms of particular cytokines. In the forefront of this interest was always the classical antiviral protein, interferon, but as well as this another cytokine, Tumour Necrosis Factor (TNF) found favour after it was demonstrated that it too was directly antiviral and synergised with IFNs. This of course created great anticipation that such immunological proteins might be useful in human therapy, both in the context of neoplasia and viral infection. A problem that restricted the clinical application of such cytokines were their severe and often disease-enhancing side-effects. This situation emphasises the need for alternative approaches to the administration of recombinant cytokines as pharmacological agents. Such an approach is through the use of specific antibodies to enhance positive functions, such as antiviral efficacy, while inhibiting side-effects.

The work of this thesis shows that particular antibodies are capable of enhancing the antiviral activity of TNF both *in vivo* and *in vitro*. Vaccinia virus is resistant to the *in vivo* actions of TNF if administered alone. TNF is effective against vaccinia virus infection when complexed to either Mab 32 or Ab 301. With Herpes simplex virus type 1 (HSV-1) TNF alone was effective in attenuating it's *in vivo* growth and the antibodies which enhanced TNF antiviral effect against vaccinia virus generally had no effect. The study of host immune factors in TNF ± Ab 301 pretreated and vaccinia virus infected mice revealed that neither natural killer (NK) cell nor cytotoxic T lymphocyte (CTL) activity was enhanced. This evidence plus the observation that the TNF/Ab 301 anti-vaccinia virus effects were present as early as day 1 post-infection suggests that an early, non-specific and probably inflammatory mechanism was restricting the growth of vaccinia virus. Studies of the direct *in vitro* antiviral effects

of TNF revealed some clues regarding the mechanism of TNF antiviral effects and the resistance of vaccinia virus to TNF compared to the sensitivity of HSV-1. It was found in studies with the antioxidant, butylated hydroxyanisole (BHA), that free oxygen radical activity was important to the resistance of L929 cells to infection with HSV-1, but not vaccinia virus. It also appears that pre-exposure to TNF sensitises these cells to the cytotoxic effects of the BHA-sensitive factor.

Of interest also was the role of the immunoregulatory cytokine transforming growth factor beta (TGF β) in vaccinia virus growth. It was found that a recombinant vaccinia virus encoding the gene for murine TGF β2 did not have its growth attenuated compared to a control virus in either thymic CBA/H mice or athymic Swiss nude mice. Also immune cell populations, namely NK cells and CTLs did not have their function affected in mice infected with VV-TGF β2. This lack of activity could be related to the TGF β property of post cell secretion latency which, without further extracellular processing, renders this molecule biologically inactive.

Abbreviations.

µg	microgram
^{0}C	degrees Celsius
Ab	Antibody
ATP	Adenosine tri-phosphate
BHA	butylated hydroxyanisole
BSA	Bovine serum albumin
Ci	Curies
cm	centimetres
CPE	Cytopathic effect
CPM	Counts per minute
CTL	Cytotoxic T Lymphocyte
DMEM	Dulbecco's minimum essential medium
DNA	Deoxyribose nucleic acid
DTH	Delayed-type hypersensitivity
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immunosorbant assay
EMCV	Encephalomyocarditis virus
EMEM	Eagle's minimum essential medium
FCS	Foetal calf serum
$Fe^{++/+++}$	Iron (ferric and ferrous)
HA	Haemagglutinin
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
H_2O_2	Peroxide

HSV-1	Herpes Simplex Virus, type 1
i.p.	intraperitoneally
i.v.	intraveneously
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kD	kilodaltons
LAK	Lymphokine activated killer
LCMV	Lymphocytic choriomeningitis virus
M	Molar (moles/litre)
Mab	Monoclonal antibody
mg	milligram
mls	millilitres
MOI	Multiplicity of Infection
MTD	Mean time to death
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
ng	nanogram
NK	Natural killer
NSB	Non-specific binding
O_2	Oxygen
O_2^-	Superoxide
OH^-	Hydroxyl radical
p.i.	post-infection
PBS	phosphate buffered saline

pfu	plaque forming units
PSN	Penicillin, streptomycin, neomycin
R	Rads
RNA	Ribose nucleic acid
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RPM	revolutions per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAE	Tris Acetate EDTA
TGF	Transforming growth factor
TK	Thymidine kinase
TNF	Tumour necrosis factor
U.V.	Ultraviolet
VSV	Vesicular stomatitis virus
VV	Vaccinia virus

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Chapter 1.

INTRODUCTION AND LITERATURE REVIEW.

1.1 The emergence of cytokines.

The 1960's was a period of intense investigation of the cellular immune response. Through these investigations came the discovery that the supernatants of lymphocyte cultures, sensitised with a specific antigen, yielded soluble factors which had properties such as lymphocyte mitogenesis and macrophage migration inhibition, which strongly suggested a role for these factors in cellular immune regulation. 1969 saw the introduction of the term lymphokine (Dumonde, *et al.*, 1969). These factors were found to affect many different target cell types emphasising their importance on the normal physiology of immune mechanisms (Waksman, 1979). With the following period of consolidation in cytokine biology came the ability, via advances in molecular biology, to better characterise the various cytokines as well as isolate their genes and produce recombinant proteins of high purity. With this greater availability of cytokines came more interest in studying the potential application of these immunological factors to disease states. The study of the role of cytokines in cancer has always been in the forefront. Since the early 1980's however, there has been increasing interest in the role of cytokines in viral infection. The discovery of a virus as the responsible agent in acquired immunodeficiency syndrome (AIDS) did much to enhance this interest. Particular cytokines with antiviral properties have been identified. Much of the work which has established the antiviral potential of some of these proteins, as well as showing the possible mechanisms involved with these antiviral properties, has been done *in vitro*.

1.2 The Antiviral Cytokines.

1.2.1 The Interferons.

The discovery of the fundamental actions of interferons (IFNs) actually pre-dated the above described emergence of interest in cytokines that occurred during the 1960's (Isaacs & Lindenmann, 1957). These proteins are released by virally infected cells and afford protection to the surrounding non-infected cells via complex mechanisms. Interferons were initially classified on the basis of cellular origin. Therefore IFNs were designated as either leucocyte interferon, fibroblast interferon or as immune interferon. Further to this, leucocyte IFN and fibroblast IFN displayed the same properties of heat and acid resistance, whereas IFN γ was acid and heat labile. On the basis of these physicochemical characteristics leucocyte IFN and fibroblast IFN were further classified as type I IFNs and IFN γ as type II. The above criteria for IFN classification were discussed and subsequently amended at a specially-convened meeting in 1980 (Stewart II *et al.*, 1980). It was decided that the old system of classification was misleading especially as cell types like leucocytes and fibroblasts were capable of secreting heterogenous mixtures of IFNs and "immune" IFN could be induced via means other than immune recognition. It was therefore recommended that IFNs should be classified on the basis of antigenic specificities, with the classifications of α, β and γ corresponding to the old classifications of leucocyte, fibroblast and immune IFNs.

The mechanisms of IFN antiviral action has been well studied. IFN strongly induces (via ds RNA) the synthesis of 2'-5'-oligoadenylate (2-5 A) synthetase. This enzyme subsequently catalyses the conversion of ATP to oligonucleotides which activate RNase L, an endogenous endonuclease which degrades both host and viral mRNA and rRNA hence stopping protein synthesis (Kerr & Brown, 1978; Zilberstein *et al.*, 1978; Baglioni, 1979). As well as 2-5 A synthetase activity IFN induces the

production of 48 and 68 kD subunits of a 110 kD protein kinase (Zilberstein *et al.*, 1978). In the presence of ds RNA the 48 kD subunit phosphorylates the 68 kD subunit which leads to the activation of intrinsic protein kinase activity. In this system there is also the phosphorylation of other substrates, for example the alpha subunit of eukaryotic protein synthesis initiation factor 2 (eIF2α; [Farrell *et al.*, 1977]). The mechanisms and complexities of IFN action on various viruses has been recently reviewed by Samuel (1991).

1.2.2 The Tumour Necrosis Factors (TNF).

Observations of TNFs actions occurred as early as the late 1800s with a physician of the time, William Coley who noticed in cancer patients regression of tumours if there was a concurrent bacterial infection. Therefore, Coley and a number of other physicians attempted to infect advanced cancer patients with certain bacteria under more controlled conditions. Infection of these patients proved difficult and when infection did take there was no way to control its severity, although some antitumour responses were seen. In 1893 Coley therefore turned to using killed bacterial preparations, most particularly a mixture of *Streptococcus pyogenes* and *Serratia marcescens* and this preparation became known as "Coley's toxins" (Coley, 1893; Coley, 1906). For a time Coley's toxin was in vogue but with the advent of alternative cancer therapies and the continuing controversy over the treatment led to the gradual diminishing of interest in its use (Old, 1985).

Although interest in the toxin therapy lost its impetus this did not impede future studies of the potential of microbial products as antitumour agents. This area of interest led to the discovery that haemorrhagic necrosis of particular mouse tumours was observed soon after the injection of filtrates from cultures of Gram-negative bacteria

cultures. This activity was subsequently identified by Shear *et al.* (1943) to be due to a polysaccharide, namely lipopolysaccharide (LPS) which is a major constituent of the Gram-negative bacterial cell wall. LPS was limited as a therapeutic agent in humans, however, because of its toxicity.

It was not until the 1970s-80s that the host-derived agent responsible for these toxin-induced antitumour effects was revealed, based on 2 distinct lines of research. The host factor responsible for the necrosis of tumours in BCG-infected and LPS-treated mice was called tumour necrosis factor (TNF; Carswell *et al.*, 1975). Another separate line of research, on cachexia in rabbits infected with *Trypanosoma brucei brucei*, led to the identification of the responsible host-derived factor. These infected animals lost more than half of their initial body mass due to hypertriglyceridaemia caused by the inhibition of the enzyme lipoprotein lipase (LPL; Rouzer & Cerami, 1980). A similar suppression of LPL was also observed in animals treated with LPS and this was caused by a transferable serum factor (Kawakami & Cerami, 1981). The LPL-inhibiting factor was subsequently purified and named cachectin (Beutler *et al.*, 1985a). The comparison of the amino-terminal sequence and immunological properties of mouse cachectin with TNF α showed that these 2 entities were in fact identical molecules (Beutler *et al.*, 1985b; Beutler & Cerami, 1989). Since this discovery many other biological activities have been ascribed to this protein and it is regarded as one of the more pleiotropic cytokines with roles in both immune and inflammatory function as well as disease pathology. The term cachectin has largely disappeared and this protein is most commonly referred to as TNF.

1.3 *In vitro* studies on the role of cytokines in viral infection.

Although interferon-induced antiviral activities had been known about for several years, the early-mid 1980s was a period of renewed interest in the interferon family of cytokines which, as earlier mentioned, could have been a consequence of the greater availability of such proteins via recombinant DNA technology. Fleischmann *et al.* (1984) carried out studies which found that both IFN α and IFN β acted synergistically with IFN-γ in inhibiting Newcastle Disease Virus replication in a murine model. From this work the authors also drew conclusions that the phenomena they saw could also be applicable to a human system. Around the same time it was found that treatment of cells with *E. coli* derived IFN γ and IFN α or IFN β_1 resulted in the potentiation of both antiproliferative and antiviral (herpes virus) activity (Czarniecki, 1984; Oleszak & Stewart, 1985). Interest in the possible antiviral action of non-interferon cytokines such as tumour necrosis factor (TNF) also evolved during this time. It was found that hTNF α selectively killed herpes virus infected, but not normal cells whereas recombinant IL-1, IL-2, IFN α and IFN γ were not cytotoxic for virus infected cells (Koff & Fann, 1986). Later, TNF α and IFN γ or β were also found to be effective in nervous tissue against infection by the neurotropic pseudorabies virus (Schijns *et al.*, 1991).

During investigations on the possible role of TNF as an inducer of IFN, Mestan *et al.* (1986) found that recombinant human TNF was antiviral against VSV, HSV and EMCV *in vitro*. The inhibition of viral proteins was found to be induced in cells treated with TNF. The investigation of the possible role of the interferons in this antiviral state also was examined. It was found that anti-IFN α and anti-IFN γ were not capable of blocking the antiviral induction by TNF, whereas anti-IFN β treatment resulted in a partial reduction of the TNF mediated effects. These workers also found that the antiviral IFN induced enzyme, 2'-5' oligosynthetase, was not enhanced with

TNF treatment, supporting the notion that IFN activity may not be important in the observed TNF induced antiviral effects. At the same time as the report by Mestan *et al.* came further support for the *in vitro* antiviral efficacy of TNF from Wong and Goeddel (1986). These authors showed that both TNF α and β were antiviral in 7860 cells against DNA (Adenovirus-2, HSV-2) and RNA (EMCV, VSV) viruses *in vitro*. As well as this the authors found that both types of TNF synergised with IFN γ against all the viruses examined in A549 cells. Again the possible induction of IFNs were considered by the authors as the mechanism by which TNF exerts its antiviral state. It was found subsequently that there was no significant IFN involvement, although, it was shown that, like IFNs, TNF induced 2'-5'-oligoadenylate synthetase, with both cytokines together inducing a stronger response than either cytokine alone in A549 cells. The observation that with A549 cells TNF or IFN γ alone were not antiviral demonstrates, however, that the upregulation of 2'-5'A synthetase activity was not a significant factor in the induction of an antiviral state in this model.

The antiviral activity of TNF and IFN γ against human immunodeficiency virus (HIV) has also been examined by Wong *et al.* (1988a) *in vitro*. In this study it was found that the pretreatment of HuT 78, normal $CD4^+$ cells and RPMI 1788 cells with TNF or IFN γ alone had marginal effects on the percentage of cells infected by HIV, as measured by the presence of the HIV core protein p24 and HIV RNA production. However, in these above-mentioned cell lines TNF in combination with IFN γ showed a dramatic synergy in the reduction of HIV infection parameters when compared to treatment with either cytokine alone. The TNF/IFN γ treatment was also found to be effective against both acutely and chronically infected HuT 78 cells. In contrast to these findings other studies have found that TNF can stimulate HIV-1 transcription in human T-cells (Israel *et al.*, 1989; Folks *et al.*, 1989) and TNF has also been found to

synergise with interleukin 6 (IL-6) in the induction of HIV-1 expression in infected monocytic cells (Poli *et al.*, 1990). It appears that the influence of effector cytokines such as TNF depends upon the target cell type, and that while this cytokine is responsible for inhibiting HIV, it may also contribute to establishing a reservoir of infection in certain tissues.

1.4 The mechanism of TNFs direct antiviral effects and the possible role of IFN β.

These initial observations stimulated considerable interest into the putative role of IFNs in the TNF-induced *in vitro* antiviral effect. Ito and O'Malley (1987) showed recombinant TNF to be antiviral in human diploid fibroblasts infected with HSV-1, HSV-2, CMV, varicella-zoster virus, VSV and EMCV. The above observed antiviral actions were completely neutralised by anti-IFN β serum, but not by anti-IFN α or IFN γ antibodies. This finding is in partial agreement with Mestan's findings but contrary to the earlier findings of Wong & Goeddel. Mestan *et al.* (1988) went onto further investigate this TNF/IFN question and found initially that TNF induces an antiviral state in HEp-2 cells with a synergistic effect elicited in the presence of low concentrations of IFN β_1 or IFN γ. These antiviral pathways were found to involve oligo-2',5'-adenylate synthetase and were sensitive to an antiserum against IFN β_1. From this observation came the hypothesis that the induction of an antiviral state by TNF was an indirect event mediated by IFN β_1. Evidence on this question was also forthcoming from Hughes *et al.*, (1988) who demonstrated that the TNF-induced potentiation of the IFN γ antiviral state in HEp-2 cells was dependent on IFN β.

Van Damme *et al.* (1987) found that IL-1α/β as well as TNF were capable of inducing an antiviral (VSV) effect on diploid fibroblasts and MG-63 osteosarcoma cells,

and again antibodies to IFN β_1 were found to neutralise this effect. In addition, this treatment induced a 26 kD protein which was shown to be IFN β_2. There was no good correlation though between the production of this protein and the antiviral effects of TNF and there was only weak neutralisation of the IL-1/TNF induced antiviral state by an antibody specific to this 26 kD protein. This evidence had these authors convinced that the β_1 form of IFN was the most likely mediator of TNF/IL-1 induced antiviral effects. This was further supported by other studies which found that IFN β antibodies were able to abrogate the TNF α,β/IL-1 induced antiviral (VSV) state in WISH cells and it was thought by these workers that the IFN involved in the antiviral pathway was not β_2. It was also found that 2-5A synthetase activity was significantly increased in cells which were treated with TNF doses which afforded antiviral protection (Ruggiero *et al.*, 1989a; Ruggiero *et al.*, 1989b). Although the evidence at the time seemed to be supporting very strongly the role of IFN β as an intermediate molecule in TNF-induced antiviral effects other evidence appeared that challenged these findings and supported the earlier work of Wong and Goeddel. Gessani *et al.* (1988) found, in studies which utilised specific IFN antibodies as well as RNA analysis, that in HeLa cells IFN β was not important to the TNF-mediated *in vitro* antiviral effect.

More evidence to support the notion that IFN β_1 is a factor in TNF induced antiviral effects *in vitro* came from work by Jacobsen *et al.* (1989) who also used VSV as a model virus. As with previous studies it was shown that TNF could induce an antiviral state in various cell lines. The difference of this study, however, was that the TNF/IFN phenomena was examined at the RNA level. It was found that after TNF treatment there was an increase in IFN β_1 specific transcripts which followed a dose response, and correlated with the TNF-induced antiviral state. Up to this time the work had concentrated on the neutralisation of the expressed IFN proteins by antibodies.

Also, molecular studies to this time had tended to focus on the expression of 2',5' adenylate synthetase transcripts after TNF/IFN treatment and not so much on the direct induction of IFN genes.

Herpes simplex viruses, particularly HSV-1, had been found to be sensitive to both interferons and TNF. Elucidation of the *in vitro* antiviral mechanisms of TNF and interferons have therefore used HSV-1 in many of these studies. Klotzbucher *et al.* (1990) found that in primary murine macrophages recombinant murine IFN γ pretreatment caused a dose dependent decrease in progeny virus and inhibition of HSV-1 protein synthesis; this was comparable to that found for IFN α/β pretreatment. Expression of the immediate early IE3 gene product ICP4 in IFN γ treated cells was found to be similar in control-infected cells, however, there was a delay in the accumulation kinetics of these genes with the IFN γ treatment. The IFN α/β treatment lead to a reduction in IE3 transcripts. Thus, with the reduction of IE3 gene products after IFN γ treatment, but no marked difference found in the RNA levels and transcriptional activity of the associated genes when compared to control cells, it was hypothesised that IFN γ could be responsible for a translational inhibition of IE3 gene products. IFN α/β treatment resulted in decreases in ICP4-RNA with a delay in the accumulation of this transcript, as well as causing the reduction of IE expression at the protein level. It was therefore thought that IFN α/β and IFN γ may have different antiviral mechanisms. This could explain the synergy seen between these interferons.

Using analysis of HSV-1 protein inhibition in Hep-2 cells Feduchi and Carrasco (1991) recently assessed the question of TNF/IFN inhibition of HSV-1 infection *in vitro*. These authors confirmed earlier findings that TNF synergises with IFN γ in inhibiting HSV-1 replication, as well as further supporting the involvement of IFN β in this process. They demonstrated that IFN β_1 added exogenously displayed antiviral

activity, but only at high doses. IFN β_2 (IL-6) did not show any anti-replication affect on either VSV or HSV at concentrations of up to 1000 U/ml. From this study the authors concluded that their findings confirmed the concept that TNF induces IFN β_1 and went on further to say that in their opinion both TNF and IFN β_1 need to be present in the culture medium to synergise with IFN γ in the inhibition of HSV-1 replication. These authors had also earlier shown that the synergistic blockade of HSV-1 replication by TNF and IFN γ occurs at a stage of infection after virus entry but before, or at the time of, the transcription of immediate early genes (Feduchi *et al.*, 1989).

Clearly there was controversy involved in the putative role of IFN β in the induction of a TNF antiviral effect. The weight of evidence does generally support the involvement of IFN β as an important factor in TNF-induced antiviral effects, although, it should be noted that all the work has been done *in vitro* and the role of TNF-induced IFN β could well be cell specific. During the study of this enigma there has not been a great deal of thought, outside the traditional IFN-induced pathways, directed towards a definitive direct effector mechanism in cytokine-induced antiviral effects, and this question is considered at some length later in this thesis.

1.5 Examination of the role(s) of cytokines in *in vivo* virus infections.

The natural next step once the *in vitro* potential of particular cytokines to inhibit viral replication and/or growth had been established was to translate these findings into animal models. In general, attempts to induce antiviral states with exogenous cytokines in animals such as mice has only been moderately successful. Klavinskis *et al.* (1989) have shown that LCMV does not respond to TNF in a mouse model, whereas IFN γ was effective but there was no synergistic effect seen if TNF and IFN γ were administered together. Other examples of the failure of cytokines to be effective

antiviral agents *in vivo* also exist (Leist & Zinkernagel, 1990; Paya *et al.*, 1990). There have been some successes, however, in mouse models, particularly with herpes viruses and IFN α (Hendricks *et al.*, 1991) as well as HSV-1 and TNF α (Rossol-Voth *et al.*, 1991). It has also been recently shown that TNF α is effective against encephalomyocarditis virus (EMCV) in BALB/c mice (Sriram *et al.*, 1991). The administration of mouse interferon to ICR mice 24 hours prior to infection with ectromelia was shown to decrease mortality as well as increase survival times (Imanishi *et al.*, 1980; Imanishi *et al.*, 1981). Shalaby *et al.* (1985) have shown also that IFN γ is effective in protecting CD-1 mice from lethal infection by EMCV. Rodriguez *et al.* (1991) have demonstrated that the inhibition of a pox virus (in this case vaccinia virus) in peritoneal and spleen cells from BALB/c mice by IFN α + β is mediated by early events post-infection, most likely, in the opinion of these authors, via a translational block. A murine retrovirus (Raucher's murine leukaemia virus) has also been shown to be responsive to the antiviral properties of IFN α (Ruprecht, 1989). This inhibition of retroviral growth followed a dose dependency and was found to synergise with AZT. The interest in this work is in the development of a small animal model to test possible therapies for HIV infection. As previously mentioned some *in vitro* studies have found that cytokines, particularly TNF as well as TGF β (Lazdins *et al.*, 1991) can enhance HIV replication in certain cell types. These findings suggest the need for caution in future clinical trials involving cytokines and HIV infected patients.

1.6 Studies of recombinant vaccinia viruses encoding cytokine genes.

Original studies were done in which the mouse IL 2 gene was inserted into a non-essential region of the vaccinia virus genome under the influence of a vaccinia virus early/late promoter. Infection of cells *in vitro* with this VV-IL 2 showed that IL

2 could be expressed in this system. When this recombinant vaccinia virus was used to infect immunodeficient Swiss Nude mice it was found that all of the infected mice survived this challenge whereas the Nude mice infected with a control virus all died approximately 10-12 days after challenge (Ramshaw *et al.*, 1987; Flexner *et al.*, 1987). The mechanism of this protective effect has since been elucidated and involves the induction of greater NK cell activity by IL 2 and the resultant increase in IFN γ production (Karupiah *et al.*, 1990a; Karupiah *et al.*, 1990b). This model therefore is another approach to the study of cytokine-induced effects on vaccinia virus, particularly *in vivo*. These findings initiated interest in exploring the effects of other cytokines in this recombinant vaccinia virus model and this topic has been recently reviewed by Ramshaw *et al.*, (1992).

1.7 The use of cytokines as antiviral agents in humans.

Several studies have been carried out examining the potential of cytokines as therapeutic agents in human viral infections. A virus that has received much attention in this regard has been hepatitis B virus (HBV). Both IFN α (Daniels *et al.*, 1989) and TNF (Sheron *et al.*, 1990) treatments were effective in some patients with chronic HBV, although, with TNF therapy there were some toxic side-effects noted. Interferon gamma has also been examined clinically in the context of hepatitis B infection (Kakumu *et al.*, 1991). IFN γ therapy did exert a significant inhibitory effect on HBV-associated DNA polymerase but it was found not to be as effective as the IFN α therapy. Another drawback of the IFN γ therapy versus IFN α was that the side-effects induced were more severe even though a lower dose of IFN γ was utilised.

A recent study by Agosti *et al.* (1992) monitored side-effects of patients with AIDS related complex (ARC) to TNF and/or IFN γ therapy. Patients from all 3

treatment groups, ie, TNF alone, IFN alone or TNF plus IFN, experienced a range of side-effects ranging from fever, constitutional symptoms (ie, fatigue, headaches and night sweats) to local reactions, although specific renal, hepatic or haematologic abnormalities were not observed. Further to these observations, measurement of HIV related parameters were also done. CD4 lymphocyte counts, β_2 microglobulin, p24 antigen, and anti-p24 antibody levels did not significantly differ during therapy. Also no significant change was seen in the concentration of HIV both in plasma and peripheral blood mononuclear cells. The pharmacologic effects of cytokines in chronic HIV infection has recently been reviewed (Poli & Fauci, 1992). In this review the relative advantages and disadvantages of certain cytokines in HIV infection are discussed. As mentioned earlier TNF can activate HIV replication in both T cells and mononuclear phagocytes. Also in T cells IL 2 provides a signal which while inducing cell proliferation also stimulates virus replication. In mononuclear phagocytes inhibition or enhancement of HIV depends on the cytokine type, for example colony stimulating factors (CSFs), IL 1, IL 3 and IL 6 will enhance HIV proliferation whereas IFN α/β will suppress HIV replication in monocyte/macrophages and T cells. TGF β will be discussed in the context of HIV infection later in this thesis. A challenge for future work on the clinical application of cytokines to HIV infection and AIDS will be to selectively enhance the antiviral functions of these proteins while nullifying the properties which promote viral proliferation.

The short plasma half-life of cytokines may prove troublesome in human studies, since large, potentially toxic doses may need to be administered. The promise held for cytokines, particularly interferons, as potential therapeutic agents has not materialised due principally to toxic side effects associated with their administration (Fent & Zbinden, 1987). Interferons are potently antiviral, however, the side-effects associated

with interferon therapy can often exacerbate disease symptoms. Like the interferons, TNF can induce a range of undesirable side-effects, for example, fever, chills, fatigue and life-threatening complications such as hypotension and hypoglycaemia, if administered in large and/or continuous doses (Spriggs *et al.*, 1988; Creagan *et al.*, 1988a; Creagan *et al.*, 1988b).

1.8 Transforming Growth Factors beta.

The name transforming growth factor (TGF) is a term which was applied to 2 separate molecules, ie TGF α and TGF β, on the basis of the nature of the assay used in their discovery and subsequent purification. The first report of TGF bioactivity was by De Larco and Todaro (1978). These workers reported that mouse 3T3 cells, which had been transformed by Moloney sarcoma virus, produced polypeptide growth factors which were named sarcoma growth factors (SGFs). This factor was secreted into the extracellular medium and induced a transformed phenotype in non-neoplastic "reader" cells for example the rat NRK 49F fibroblast. One result of this transformed phenotype was that the cells acquired an anchorage independence *in vitro* which allowed these cells to grow in soft agar. With the introduction of acid-ethanol to extract transforming peptides from tumour cells themselves (Roberts *et al.*, 1980) the term transforming growth factor was introduced as it was realised that peptides with similar properties could be isolated from a very diverse range of tumour cells. Furthermore, TGF activity could also be isolated from almost all normal mouse tissues (Roberts *et al.*, 1981). With the use of high-pressure liquid chromatography (HPLC) and the NRK 49F cell line in soft agar assays 2 principal TGFs were isolated. The peptides, which competed with epidermal growth factor (EGF) for receptor binding, but did not need extra EGF for cell growth promotion, were termed TGF α. Peptides which did not compete with

EGF for receptor binding and did require EGF for the promotion of cell growth in soft agar were termed TGF β (Anzano *et al.*, 1982; Roberts *et al.*, 1983). In addition to these findings it was shown that EGF and TGF α together could not promote cell growth in the NRK system as they relied on the concomitant presence of TGF β for this activity (Anzano *et al.*, 1982). With this discovery it was then possible to achieve total purification and characterisation of TGF β. On the basis of studies on a variety of tissue types, this peptide was found to be a homodimer of 25.0 kD which gave 2 monomeric subunits of 12.5 kD upon reduction, with identical amino-terminal amino acid sequences (Roberts & Sporn, 1988).

It is now known that TGF β exists as 3 distinct molecular forms in mammals, namely TGF β1, TGF β2 and TGF β3. A fourth subtype has been found in birds and a fifth in amphibians (TGF β4, TGF β5) and it appears that these molecules may share some activities with mammalian forms (Jakowlew *et al.*, 1988; Kondaiah *et al.*, 1990; Roberts *et al.*, 1990). The β2 and β3 forms of TGF are initially synthesised as larger precursor molecules and processed to yield 12.5 kD monomers in similar fashion to TGF β1. The processing of TGF β1 is not perfectly understood but is known to involve the cleavage of a signal sequence, glycosylation and mannose-6-phosphorylation of the precursor, cleavage of the carboxy-terminal 12.5 kD (112 amino acid) monomer from a 390 amino acid precursor and disulphide isomerisation. TGF β is secreted from cultured cells as a form that will not bind the receptor and is therefore biologically inactive (Barnard *et al.*, 1990). Activation of this latent TGF (ie, production of a form that will bind to the TGF receptor) can be achieved by several physico-chemical treatments including heat, acidification and the use of chaotropic agents such as urea (Lawrence *et al.*, 1985). The enzyme plasmin has recently been shown to be capable of activating latent TGF and, therefore, is a likely candidate for the physiological

activation of TGF (Lyons *et al.,* 1990).

Active TGF β constitutes 2 identical 112 amino acid (12.5 kD) polypeptide chains joined via 9 disulphide bonds (referred to above). The amino acid sequences of the active TGF β chains displays high homology between the β1, 2 and 3 forms and as could be expected show very similar characteristics in many assays. The human TGF β1 chain is approximately 71 % homologous to that of TGF β2 and 77 % homologous to TGF β3. TGF β2 is approximately 80 % homologous to TGF β3. The positions of the 9 cysteine residues are precisely conserved. TGF β primary structure is also highly conserved between different animal species and as may be expected show high degrees of cross-species activity (Derynck *et al.*, 1988; Meager, 1991).

TGF β has been shown to be a generally immunosuppressive molecule, effecting various arms of the immune response. Examples of this include, the antiproliferative effects on B and T lymphocytes (Kehrl *et al.*, 1991a), inhibition of CTL development (Ranges *et al.*, 1987), the inhibition of immunoglobulin (Ig) production in human lymphocytes (Kehrl *et al.*, 1991b; Warmold *et al.*, 1992), and inhibition of B cell activation and antigen-specific B cell-Th cell physical interactions (Bartlett *et al.*, 1991). HIV has been studied in terms of TGF β antiviral activity, and the reports on its effectiveness have varied. A report has shown TGF β to be effective in the restriction of HIV growth (Poli *et al.*, 1991), whilst others have found that TGF β enhances the growth of HIV (Lazdins *et al.*, 1991; Lazdins *et al.*, 1992). While TGF β does not appear to be a useful cytokine for augmenting immune responses, the investigation of TGF β as an immunoregulator is essential, particularly in it's relationships with other cytokines and effector molecules.

1.9 Aims of this Thesis.

The work of this dissertation will examine further the antiviral properties of cytokines, particularly from an *in vivo* perspective. An emphasis of much of the work will be on the enhancement of the antiviral actions of human TNF α by specific antibodies, one monoclonal and the other polyclonal. Such antibodies are of particular interest as potential agents to increase the therapeutic index of cytokines used in human disease. The enhancing properties of these antibodies allowed a more comprehensive study of TNF antiviral activities *in vivo*, particularly in a vaccinia virus infection model. Because of the well documented antiviral synergy with TNF, IFN γ was also studied in the context of TNF antibody enhancement phenomenon. While much of the data concerns vaccinia virus infection it was also believed to be important that other viruses be considered in the study of TNF's enhanced *in vivo* antiviral efficacy. Therefore, work was also done using essentially similar models on herpes simplex virus 1 (HSV-1). HSV-1 also became an important tool in *in vitro* investigations on the direct antiviral effects of TNF plus antibodies.

This thesis also addresses the potential activities of transforming growth factor beta (TGF β) in vaccinia virus infection. The approach used for this investigation was to construct a recombinant vaccinia virus containing the TGF β gene. This strategy resulted in a virus capable of producing its own TGF β, thereby, allowing investigations on the direct regulation of virus activity by this protein.

Chapter 2.

General Materials and Methods.

Mice.

Female CBA/H, BALB/c, C57/B6 and outbred pathogen-free Swiss Nude mice between the ages of 6-8 weeks were obtained from the animal breeding establishment of the John Curtin School of Medical Research.

Viruses.

i) *Vaccinia virus.*

The vaccinia virus used in the experiments with recombinant TNF and/or mIFN-γ (unless otherwise stated) contained both the HSV thymidine kinase gene (TK^+) and the influenza haemagglutinin gene (VV-HA-TK). This virus was constructed from a previously described recombinant, VV-PR8-HA6 (Andrew *et al.*, 1986). The HA gene was from influenza A/PR/8/34 virus and the L929 cell-adapted WR vaccinia strain (VV-WR-L929: Wokatsch, 1972) was used as the parent virus for the construction of the recombinants. The arrangement of the recombinant HA gene in the vaccinia virus genome is described by Ramshaw *et al.*, (1987).

The construction of the thymidine kinase negative (TK^-) recombinant vaccinia virus encoding TGF β2 is detailed in chapter 8.

Virus stocks were propagated in CV-1 cells infected with a MOI* equal to 0.01 of the relevant vaccinia virus. This procedure was carried out in 2.0 litre acid-washed Schott roller bottles. The titrated, aliquoted stocks were stored in sterile vials at -70^{0}C until required. The stock virus was diluted in 2.0 mls of gelatin (0.5 %) saline to the desired concentration and sonicated at 50 watts for 5 x 2 second bursts using a Branson

Sonifier (Branson Sonic Power Company, Danbury, CT., USA).

* MOI (multiplicity of infection) is the number of virus plaque forming units per cell.

ii) *Herpes Simplex Virus type 1.*

Herpes simplex virus type 1 strain SC16 (HSV-1, SC16: Field *et al.*, 1979) was provided by Dr. A. Simmons of the Institute of Medical and Veterinary Science, Adelaide, South Australia. Stocks of the virus were propagated in Vero (green monkey kidney) cells infected at a MOI of 0.1 for 48 hours. After this period the infected Vero cells were washed/scraped into the culture media and then sonicated (as described above). The titrated stocks were then aliquoted and stored at -70^{0}C until required.

Cell lines.

All cell lines were maintained at 37^{0}C in a humidified atmosphere containing 5 % CO_2. Cells at a concentration of 1.0-2.0 x 10^6 cells were seeded into 80 cm^3 or 175 cm^3 flasks (Nunclon) for routine passage. 143 B cells (Rhim *et al.*, 1975) were maintained in DMEM (Gibco, NY, USA) supplemented with 5 % heat inactivated (56^{0}C for 30 minutes) FCS (Cytosystems, Sydney), 145 mg/litre glutamine, and antibiotics (penicillin, streptomycin and neomycin). The green monkey kidney derived cell lines Vero (ATCC, CCL 81) and CV-1 (Jensen *et al.*,1964) were maintained in RPMI (Cytosystems, Sydney) supplemented with 5 % heat inactivated FCS, 5.0 mls 5 % sodium bicarbonate and antibiotics. L929 cells (H-2^k), a mouse fibrosarcoma derived from C3H mice (Sanford *et al.*, 1948) and the mink lung fibroblast cell line, Mv-1-Lu (ATCC, CCL 64) were also maintained under these media conditions. The human cervical carcinoma cell line, HeLa (Gey *et al.*, 1952) was maintained in EMEM

(Cytosystems, Sydney) supplemented with 5 % heat-inactivated FCS and antibiotics.

All of the above cell lines, with the exception of 143 B cells, when confluent (3-4 days) were treated with 0.1 % trypsin plus EDTA/versene to remove the cells from the surface of the flask. 143 B cells were treated with 0.05 % trypsin plus EDTA/versene. All of the cell lines, once the trypsin/versene had been added, were subjected to a short period at 37^{0}C (5-10 minutes) after which the cells were resuspended in medium plus FCS, washed once and re-seeded as described above.

The non-adherent cell line YAC-1 (H-2^a) which originated from a Moloney leukaemia virus-induced lymphoma in A/Sn mice (Kiessling *et al.*, 1975) was maintained in DMEM plus 10 % heat inactivated FCS, supplemented with 5mM sodium pyruvate (Gibco, NY), 145 mg/litre glutamine, 5mls 5 % sodium bicarbonate, antibiotics (PSN) and 0.05% 2-mercaptoethanol. The cells were passaged every 3-4 days through the wells of a 24 well Linbro flat-bottomed plate (Flow Laboratories, McLean, VA). These cells were kept at 37^{0}C in a humidified atmosphere containing 5 % CO_2.

Antibodies.

The production of the specific human TNF monoclonal antibodies has been described previously (Rathjen *et al.* 1991). Monoclonal antibody 32 (Mab 32) is of the IgG2bK isotype and has been found not to inhibit TNF cytotoxicity on WEHI-164 cells *in vitro* (Rathjen *et al.*, 1991; Rathjen *et al.*, 1992).

The mapping of overlapping hTNF peptides with Mab 32 has recently shown that the regions 1-10, 40-42, 44-46, 107-114 and 127-130 are the areas in exposed loop structures of the TNF molecule to which Mab 32 binds (Rathjen & Aston, in press).

The polyclonal antibody (Ab 301; Peptide Technology, Sydney) was raised in sheep challenged with a peptide sequence, in Freund's adjuvant, from human TNF to

which Mab 32 binds.* Pre-immune sera were collected from sheep challenged with Freund's adjuvant alone.

The preparation of univalent Fab' fragments from Mab 32.

Mab 32 was first digested with agarose immobilised papain (Pierce) according to the manufacturer's instructions. The Fab' region was separated from the Fc antibody fragments by a protein A-Sepharose affinity column (Pharmacia). The Fab' fraction was then checked for size by SDS-PAGE analysis and the binding properties confirmed via radioimmunoassay with ^{125}I-TNF. The preparations of Fab' fragments were found to be essentially endotoxin free as determined by a Sigma (St.Louis, USA) kit method.

Cytokines.

Recombinant human tumour necrosis factor alpha (hTNF) was supplied by Bissendorf (Germany). The TNF was yeast derived with a specific activity of 3.2×10^7 units / mg as determined by a L929 (+ mitomycin C) cytotoxicity assay. Purity of this cytokine was greater than 97 %.

E. coli-derived murine gamma interferon (mIFN γ) was provided by Dr. G. Adolf, Boehringer Ingleheim, Vienna. The specific activity of this cytokine was 1.0×10^7 units /mg as determined by antiviral activity induced in mouse L-cells to encephalomyocarditis virus (ECMV).

* For specificity, see Rathjen & Aston (1993); Bioinorganic and Medicinal Chemistry Letters. **3**:457-462.

Chapter 3.

The Enhancement of the *in vivo* Antiviral Efficacy of human Tumour Necrosis Factor alpha (hTNF α) by Antibodies in Vaccinia Virus Infection.

INTRODUCTION.

There has been much interest recently on the potential antiviral properties of cytokines. This interest increased with reports that TNF α/β and IFN γ were directly antiviral *in vitro* in a number of cell lines and for a variety of RNA and DNA viruses. It was also found in this system that TNF and IFN together were synergistic in their direct antiviral effects (Wong & Goeddel, 1986; Mestan *et al.*, 1986). These observations resulted in a more intense pursuit for the application of TNF/IFN mediated antiviral effects *in vivo*. There has been some success in mouse models with the administration of TNF to mice infected with HSV-1 (Rossol-Voth *et al.*,1991) and encephalomyocarditis virus (EMCV; Sriram *et al.*,1991). TNF however has not been uniformly effective as an antiviral agent *in vivo*. Work by Paya *et al.* (1990) has demonstrated, that although TNF administration did inhibit Theiler's virus-induced demyelination there was no inhibition of virus replication in the central nervous system by TNF. Another study using a specific antibody to block endogenous murine TNF activity found that this approach had no effect on lymphocytic choriomeningitis virus (LCMV) viral titres in the liver or LCMV-induced disease progression (Leist & Zinkernagel, 1990). Further to this a study by Klavinskis *et al.*, (1989) showed that while IFN γ induced an antiviral effect on LCMV in mice, TNF did not and there was no antiviral synergy when the 2 cytokines were co-administered. IFN has been shown to be an effective antiviral agent in previous studies. The treatment of ICR mice with mouse interferon (L cell or brain) 24 hours before challenge with ectromelia virus

resulted in both decreased mortality rates and increased survival times (Imanishi *et al.*, 1980; Imanishi *et al.*, 1981).

Recent studies with cytokines such as IFN α and TNF α in humans infected with Hepatitis B virus have shown some encouraging results for the possible application of cytokines as clinical antiviral therapies, although cytokine-induced side effects were also observed (Daniels *et al.*, 1989; Sheron *et al.*, 1990). The initiation of secondary pathologies by cytokines is well documented (Fent & Zbinden, 1987: Spriggs *et al.*, 1988; Creagan *et al.*, 1988) and is the major drawback to using cytokines such as TNF therapeutically. One possible strategy to overcoming this problem is to use specific antibodies to epitopes of cytokines and thereby modify some of their activities. Rathjen *et al.*, (1991; 1992) have recently described a monoclonal antibody against hTNF which selectively enhances anti-tumour effects but blocks the pro-coagulant activity of TNF in cultured endothelial cells.

This chapter examines the enhancement of the antiviral efficacy of hTNF in mice challenged with vaccinia virus by both the above mentioned monoclonal antibody and a polyclonal antibody specific to the same region of TNF recognised by the monoclonal antibody.

MATERIALS and METHODS.

Treatment of mice with human TNF alone or in complex with monoclonal antibody 32 (Mab 32) or Fab fragments followed by infection with vaccinia virus.

CBA/H mice were injected intraperitoneally (i.p.) with either PBS or doses of hTNF (Bissendorf, Germany) between 2-20 μg. For experiments with monoclonal antibody 32 (Mab 32, 5.0 mg/ml: Peptide Technology, Sydney) 2 μg hTNF was

complexed with 0.3 mg, 0.5 mg, 1.0 mg or 2.0 mg of Mab 32. The TNF and Mabs were mixed and left to stand for 1 hour at room temperature before administration. The control Mabs (25 and 47) were only used at a concentration of 0.5 mg with 2 μg hTNF.

Fab fragments were used at concentrations of 0.25 mg or 0.50 mg in complex with 2 μg hTNF. As with the Mab plus hTNF treatments the Fabs were also left for 1 hour at room temperature before intraperitoneal administration.

Twenty-four hours after these treatments, the mice were injected i.p. or i.v. with 10^7 or 10^6 plaque forming units (pfu) of a vaccinia virus WR strain.

Treatment of mice with human TNF plus Ab 301 and / or murine gamma interferon (mIFN-γ) followed by vaccinia virus infection.

The protocol for this experiment was similar to that described above except for the following differences. The antibody (Ab 301, 5.0 mg/ml; Peptide Technology, Sydney) complexed to TNF was a polyclonal (described in chapter 2). This antibody was diluted 1:50 before mixing with 6 μg TNF. Treatments were administered by i.p. injection as follows: 1) hTNF plus Ab 301; 2) 10^5 U mIFN-γ and 3) combined treatments with TNF and Ab 301 plus mIFN-γ. In the latter treatment the mIFN-γ was not mixed with the TNF:Ab 301 complex until after the 1 hour room temperature incubation of TNF with the antibody. Mice were injected 24 hours later with 10^6 pfu vaccinia intravenously (i.v.). This protocol was also followed for the experiments using BALB/c and C57/B6 mice.

Measurement of vaccinia virus growth.

Mice were sacrificed 4 days post-infection (unless otherwise indicated) and the ovaries, spleens and/or lungs aseptically removed. As vaccinia grew to high titres in the ovaries these organs were chosen to monitor virus growth in the Mab 32 studies. The organs were homogenised in 1.0 ml of phosphate-buffered saline (PBS) afterwhich 100 µl of homogenate was incubated at 37°C for 30 minutes in 0.1% trypsin. Trypsinisation was stopped by the addition of heat-inactivated foetal calf serum (FCS; Cytosystems, Sydney). Samples were then serially diluted 10 fold in a HEPES buffered saline solution containing 0.5% gelatin and the dilutions absorbed for 60 minutes at 37°C in a humidified atmosphere containing 5.0% CO_2 onto a confluent monolayer of 143B human osteosarcoma cells in Linbro 6 well plates (Flow Laboratories, McLean, VA). The cells had been seeded into the L-6 plates the previous day in Autopow media (Flow Laboratories, North Ryde) supplemented with 5 % heat inactivated FCS, 145 mg/litre L-glutamine, 20 mls 5 % sodium bicarbonate, 1.5 mls 1.0 M hydrochloric acid (HCl) and PSN (Penicillin, streptomycin, neomycin). After the 1 hour virus absorption period the cells were overlaid with Autopow containing 5.0% heat-inactivated FCS. Forty-eight hours later the Autopow was removed and the cells stained with 0.1% (w/v) crystal violet in 20% (v/v) ethanol. The plates were air dried and the plaques counted. The $\log_{10}$ virus concentration was then calculated and statistical significance determined by unpaired Student's t-test.

It should be noted that the virus plaque assay technique is limited to the measurement of titres greater than two logs. Therefore individual titrations less than two logs were counted as 1.8 logs of virus.

Radioimmunoassay for hTNF binding to Mab 32.

Human TNF was iodinated (^{125}I; Amersham, 100mCi/ml) via the lactoperoxidase method (Aston *et al.*, 1985). ^{125}I-TNF was diluted and adjusted to 20 000 CPM per 50 µl. Mab 32 was initially diluted 1/50 and then serially diluted by a factor of log 5 (1/50-1/156 250). Fifty microlitres of each dilution was dispensed into duplicate tubes, with 50 µl of PBS being added to the non-specific binding (NSB) tubes. To each of these tubes, as well as the total count tubes, 50 µl of the diluted ^{125}I-TNF was added. These tubes were incubated overnight at 4^{0}C. The next day 100 µl of second antibody coated cellulose (SAC) cells (Pharmacia) were added to all tubes except the total count tubes. The tubes were then incubated for 20 minutes at room temperature, subsequently washed once with 1.0 ml of PBS, centrifuged (Beckman, TJ-6) at 2500 rpm for 5 minutes and the radioactivity of the SAC cell pellet counted in a Packard Auto-Gamma counter. The NSB was subtracted from each value and the mean CPM calculated for each dilution. The binding curve for each Mab 32 concentration tested is shown in Figure 3.1.

* The experiments performed in this chapter were all done at least twice unless otherwise indicated. Please note that (n =) refers to the number of animals or samples in an experimental or control group.

Treatment of CBA/H mice with hTNF alone.

Table 3.1 shows the vaccinia virus titres in the ovaries, lungs and spleens of CBA/H mice that had been treated with 10 or 20 μg of TNF alone at the same time as infection. The doses of TNF used did not have an effect on the growth of the virus, with the exception of a small but statistically significant increase in the vaccinia titres found in the spleen.

TABLE 3.1 Mean concentration of vaccinia virus in the organs of CBA/H mice treated with hTNF 0 hours before infection (10^7 pfu/mouse, i.p.).

	Log_{10} Virus Titre (pfu/ml) ± SEM		
Organ	**PBS (n=7)**	**10 μg hTNF (n=3)**	**20 μg hTNF (n=4)**
Spleen	2.79 ± 0.06	3.41 ± 0.06+	3.29 ± 0.07+
Lungs	3.04 ± 0.04*	3.05 ± 0.03	2.75 ± 0.15
Ovaries	8.89 ± 0.02	8.94 ± 0.02	8.75 ± 0.06

* (n=2)

+ $p < 0.001$ versus titre of the PBS treatment in the spleen.

Complexing hTNF to a monoclonal antibody (Mab 32) enhances the antiviral efficacy of this cytokine.

Figure 3.2 shows the titre of vaccinia virus in the ovaries collected from CBA/H mice 4 days after infection with 10^6 plaque forming units (pfu) of vaccinia. Twenty-four hours prior to infection the mice were treated with 2 μg of TNF either alone or combined with various doses of monoclonal antibody 32 (Mab 32). The binding of ^{125}I-TNF to the doses of Mab 32 used in this experiment are shown in Figure 3.1. The results outlined in Fig. 3.2 show that the TNF treatment alone did not significantly decrease the titre of vaccinia virus in the ovaries when compared to the control PBS treatment. With increasing Mab 32 concentration, however, there was a dose dependent decrease of viral titre in the ovaries, with 2 μg of TNF in complex with 0.5 mg Mab 32 displaying the greatest enhancement of antiviral activity when compared to TNF treatment alone. With further increases in the Mab 32 concentration enhancement of the antiviral effect was less pronounced. Ovaries from mice treated with Mab 32 alone contained an almost identical concentration of vaccinia as ovaries from PBS pre-treated mice. Two other hTNF monoclonal antibodies (Mabs 25 and 47) did not show an enhancement of antiviral activity when complexed to 2 μg TNF at the same concentration as optimal for Mab 32 (table 3.2).

Enhancement of TNF antiviral activity by Mab 32, as measured by virus titration in the ovaries, was also seen if the mice had been infected with 10^7 pfu of vaccinia (data not shown).

TABLE 3.2 Mean growth of vaccinia virus in the ovaries of CBA/H mice treated 24 hours before infection (10^6 pfu/mouse, i.v.) with hTNF plus Mabs 25 or 47.

Treatment	Log_{10} virus titre (pfu/ml) ± SEM
PBS (n=4)	8.03 ± 0.04
2 μg TNF + 0.5 mg Mab 25 (n=5)	7.48 ± 0.22
2 μg TNF + 0.5 mg Mab 47 (n=5)	8.10 ± 0.11

For both Mab treatments plus TNF there was no significant decrease in virus titre ($p > 0.05$) versus PBS. Mabs 25 and 47 were derived from the same panel of monoclonal antibodies as Mab 32 (see page 20).

With the finding of optimal enhancement of TNF it was then investigated to see whether increasing or decreasing the dose of TNF in the ratio of 2 μg TNF : 0.5 mg Mab 32 would further enhance the antiviral effects shown in Figure 3.2. As outlined in Figure 3.3 a dose of 1.0 μg TNF with the relevant amount of Mab 32 showed no decrease compared to PBS treatment. Notable decreases in virus titre were evident with the 2, 4 and 6 μg plus Mab 32 treatments but as TNF dose increased the effects were less pronounced in much the same way as seen with increasing Mab 32 doses in the presence of 2 μg TNF (Fig. 3.2). It appears that this enhancing effect is confined to a narrow TNF and Mab 32 dose window.

The complex of TNF and univalent Fab' fragments of Mab 32 were also tested to investigate the possible role of Fc region effects and bivalency (Table 3.3). At a concentration of 0.25 mg Fab'32 (plus TNF) there was no significant decrease in ovarian viral titre when compared to the PBS treatment. Treatment with 0.5 mg Fab'32

plus TNF did cause a decrease in virus concentration but this was not as pronounced as that found with the complex of TNF with intact Mab 32.

TABLE 3.3 The mean growth of vaccinia virus in the ovaries of CBA/H mice treated with hTNF in complex with univalent Fab fragments, derived from Mab 32, twenty-four hours before infection with 10^6 pfu vaccinia virus/mouse, i.v.

Treatment	**Log_{10} virus titre (pfu/ml) ± SEM**
PBS (n = 4)	7.31 ± 0.24
2 µg hTNF + 0.5 mg Mab 32 (n = 5)	6.29 ± 0.24 *
2 µg hTNF + 0.25 mg Fab'32 (n = 5)	7.03 ± 0.20
2 µg hTNF + 0.5 mg Fab'32 (n = 5)	6.52 ± 0.27 +

* Significantly lower titre (p = 0.022) compared to the PBS treatment.

+ Virus inhibition at a significance level of p = 0.068 versus PBS treatment.

Complexing TNF to a specific polyclonal antibody (Ab 301) enhances the antiviral efficacy of TNF.

Table 3.4a) shows vaccinia virus titres in the ovaries of CBA/H mice treated following the same protocol as with Mab 32 above, except that in this case the antibody to which TNF was complexed was a polyclonal antibody (Ab 301). This sheep antibody was raised against a TNF peptide to which Mab 32 binds. Mice treated with TNF in complex with Ab 301 showed a greatly reduced titre of vaccinia virus in the

ovaries, confirming the earlier results with the monoclonal antibody and showing, via a different approach, the presence of an "enhancing" epitope on TNF. This effect was optimal at a dose of 6 μg TNF. As with Mab 32 increasing TNF concentration in the presence of Ab 301 resulted in less pronounced antiviral effects (data not shown). The control treatment consisting of 6 μg hTNF mixed with a 1/50 dilution of pre-immune sera (table 3.4b) did not show a significant decrease in comparison to the 6 μg hTNF alone treatment. The difference between the PBS treatment and the TNF alone treatment was also not significant. Pretreating mice with 10^5 U mIFN γ, however, resulted in a significant decrease in virus titre (table 3.4a).

TABLE 3.4a) Mean growth of vaccinia virus in the ovaries of CBA/H mice treated 24 hours before infection (10^6 pfu/mouse, i.v) with TNF plus antibody 301 alone or murine gamma interferon alone.

Treatment	**Log_{10} virus titre (pfu/ml) ± SEM**
PBS (n=5)	8.20 ± 0.29
6 μg TNF + 1/50 Ab 301 (n=4)	5.93 ± 0.33 *
10^5 U mIFN-γ (n=5)	5.66 ± 0.35 *

* $p \leq 0.001$ versus PBS treatment.

TABLE 3.4b) Mean growth of vaccinia virus in the ovaries of CBA/H mice treated 24 hours before infection (10^6 pfu/mouse, i.v) with TNF alone or TNF with pre-immune serum.

Treatment	Log_{10} virus titre (pfu/ml) ± SEM
PBS (n = 5)	8.17 ± 0.15
6 μg TNF alone (n = 4)	7.66 ± 0.29
6 μg TNF + 1/50 Pre-immune sera (n = 5)	7.41 ± 0.24

*The above described data were the results of a single control experiment.

The enhancement of TNF antiviral activity by Ab 301 is not restricted to administration 24 hours before infection.

The enhancement of the antiviral effects of TNF by Ab 301 was shown to also be effective if administered at timepoints other than 24 hours prior to infection. Figure 3.4 demonstrates that a significant antiviral effect is produced even when the TNF plus Ab 301 is administered 72 hours before infection. Administration of TNF plus Ab 301 at the 48, 24 and 0 hour timepoints also resulted in significant reductions of vaccinia virus growth compared to the non-TNF treated controls. There were no significant differences between the TNF plus Ab 301 treatments.

Note that at 0 hours before vaccinia infection doses of 10 and 20 μg TNF alone (table 3.1) were not effective in decreasing vaccinia virus titres in the ovaries whereas 6 μg plus Ab 301 was effective in significantly reducing vaccinia titres at this time of administration.

Human TNF in complex with Ab 301 administered with murine gamma interferon (mIFN-γ) leads to a synergistic antiviral effect *in vivo*.

The antiviral effects of combined therapy with TNF and Ab 301 plus murine gamma interferon (mIFN γ) in CBA/H mice are illustrated in Table 3.5. TNF plus Ab 301 was potently antiviral, as was the murine IFN γ treatment. The combination of TNF plus Ab 301 with mIFN γ, however, displayed an even greater antiviral efficacy when compared to either the TNF plus Ab 301 or mIFN γ treatments alone. This enhanced antiviral effect was in contrast to that observed when TNF alone was combined with mIFN-γ (Figure 3.5). There were also differences seen when TNF plus Ab 301 was administered 4 hours before mIFN-γ and vice versa. There was no significant difference noted between the TNF plus Ab 301/mIFN-γ same time treatment and the treatment of IFN administered 4 hours prior to TNF plus Ab 301. The therapy of TNF plus Ab 301 administered 4 hours before IFN resulted in a significantly higher titre of vaccinia virus in comparison to the IFN before TNF plus Ab 301 treatment. The TNF plus Ab 301 before IFN treatment was not significantly different to the TNF plus Ab 301 with IFN (same time) treatment.

TABLE 3.5 Mean growth of vaccinia virus in the ovaries of CBA/H mice treated 24 hours before infection (10^6 pfu/mouse, i.v) with TNF plus Antibody 301 and gamma interferon together.

Treatment	Log_{10} virus titre (pfu/ml) ± SEM
PBS (n=5)	8.20 ± 0.29
6 μg TNF + 1/50 Ab 301 and 10^5 U mIFN-γ (same time: n=5)	<3.14 ± 0.84 +
6 μg TNF + 1/50 Ab 301 4 hours before 10^5 U mIFN-γ (n=5)	4.90 ± 0.37 *
10^5 U mIFN-γ 4 hours before 6 μg TNF + 1/50 Ab 301 (n=5)	<2.88 ± 0.68 +

TNF plus Ab 301 with mIFN-γ administered at the same time gave a significantly lower titre than both the TNF plus Ab 301 alone ($p = 0.027$) and mIFN-γ alone ($p = 0.024$) therapies (SEE TABLE 3.4a).

* $p = 0.031$ versus the treatment of IFN administered 4 hours before TNF plus Ab 301.

+ Three from five mice in these groups had no detectable virus growth, ie, < 2.0 logs.

Table 3.6 shows a similar study done in BALB/c mice. In this mouse strain there was a distinct lack of antiviral activity found with either of the cytokine treatments which were effective in CBA/H mice, namely 6 μg TNF in complex with Ab 301 alone and mIFN-γ alone. The combination of 6 μg TNF plus Ab 301 with mIFN-γ did, however, result in a significant reduction of vaccinia virus in the ovaries when compared to the TNF plus Ab 301 treatment or the mIFN-γ treatment.

In PBS treated C57/B6 mice (Table 3.7), vaccinia virus grew to only 6.5 logs, whereas in similarly treated CBA/H and BALB/c mice more than 8.0 logs of virus were present in the ovaries at 4 days post infection. In C57/B6 mice, treatment with TNF alone had no effect against vaccinia but when in complex with Ab 301, a small, but significant enhancement of the antiviral effect was seen. Murine IFN-γ was considerably more effective in reducing virus titres than the combined therapy of TNF plus Ab 301. The antiviral effect of the combination of TNF plus Ab 301 with mIFN-γ was not significantly different to mIFN-γ alone nor was there a statistical difference found when this treatment was compared to the TNF plus Ab 301 therapy.

TABLE 3.6 Mean growth of vaccinia virus in the ovaries of BALB/c mice treated with cytokines 24 hours before infection (10^6 pfu/mouse, i.v).

Treatment	**Log $_{10}$ virus titre (pfu/ml) ± SEM**
PBS (n=5)	8.17 ± 0.28
6 μg TNF (n=4)	8.02 ± 0.28
6 μg TNF + 1/50 Ab 301 (n=5)	8.05 ± 0.36
10^5 U mIFN-γ (n=4)	8.16 ± 0.47
6 μg TNF + 1/50 Ab 301 and 10^5 U mIFN-γ (n=3)	6.75 ± 0.09 *

* Significantly lower titre than both the TNF plus Ab 301 alone (p = 0.035) and mIFN-γ alone (p = 0.05) therapies.

TABLE 3.7 Mean growth of vaccinia virus in the ovaries of C57/B6 mice treated with cytokines 24 hours before infection (10^6 pfu/mouse, i.v).

Treatment	**Log $_{10}$ virus titre (pfu/ml) ± SEM**
PBS (n=5)	6.50 ± 0.39
6 μg TNF (n=5)	6.56 ± 0.17
6 μg TNF + 1/50 Ab 301 (n=5)	5.88 ± 0.24 *
10^5 U mIFN-γ (n=5)	4.14 ± 0.56 +
6 μg TNF + 1/50 Ab 301 and 10^5 U mIFN-γ (n=5)	5.21 ± 0.41

* Significant decrease in viral titre compared to TNF alone ($p = 0.048$).

+ Significantly lower ($p = 0.021$) titre in comparison to TNF plus Ab 301 therapy.

The enhanced antiviral efficacy of TNF with Mab 32 are seen as early as day 1 post infection.

Table 3.8 describes results of an experiment which examines the kinetics of vaccinia virus growth in TNF ± Mab 32 treated mice between days 1 and 4 post infection. At every timepoint after infection there is a notable decrease in virus titre in the TNF plus Mab 32 treated mice in comparison to the PBS control mice. This is also found when the TNF plus Mab 32 results are compared to those of the TNF alone treatments with the exception of the day 2 TNF alone result which also shows a decrease in virus titre. There were no significant differences found between the titres of the PBS treated mice and the TNF alone treated mice at any timepoint post infection.

TABLE 3.8 Time course of the growth of vaccinia virus in the ovaries of mice (CBA/H) treated with hTNF either alone (n=3) or with Mab 32 (n=3) twenty-four hours before infection (10^6 pfu/mouse, i.p.).

	Log_{10} Virus Titre (pfu/ml)		
Days (p.i.)	**PBS (n=3)**	**4 µg TNF**	**4 µg TNF + 1.0 mg Mab 32**
1	3.22 ± 0.22	3.23 ± 0.19	2.32 ± 0.30**
2	6.62 ± 0.50	5.56 ± 0.31	5.80 ± 0.06
3	7.50 ± 0.30	7.54 ± 0.27	6.43 ± 0.30+
4	8.82 ± 0.04	8.95 ± 0.29	6.99 ± 0.01*+

* n=2; the third mouse had no detectable growth of virus, ie, < 2.0 logs.

** p = 0.06 versus day 1 TNF alone treatment.

+ p < 0.05 versus TNF alone treatments of the same day post infection.

SUMMARY.

This chapter details the enhancement of the antiviral efficacy of human TNF by a monoclonal antibody and a specific polyclonal antibody in vaccinia virus infected mice. Treatment of mice with TNF alone either before or at the same time as infection with vaccinia virus resulted in little or no effect on *in vivo* virus growth. Antiviral activity was displayed, however, if TNF was in complex with either Mab 32 or Ab 301 at optimal doses. This effect was so potent that administration of TNF with Ab 301 3 days prior to infection still resulted in a significant reduction in vaccinia virus growth. Further to this, if the TNF, Ab 301 complex was co-administered with murine IFN γ there was a further significant reduction in vaccinia virus growth when compared to the individual therapies. TNF administered alone with IFN γ did not display any additional antiviral effects. Data presented in this chapter also suggest that the induction of an antiviral effect by TNF plus enhancing antibodies occurs within the first 24 hours following infection (this is dealt with in greater detail in chapter 5). It therefore appears that specific antibodies can enhance TNF's antiviral activity.

IFN-γ was also potently antiviral. The response to the above described antiviral therapies varied according to the genetic background of the mice.

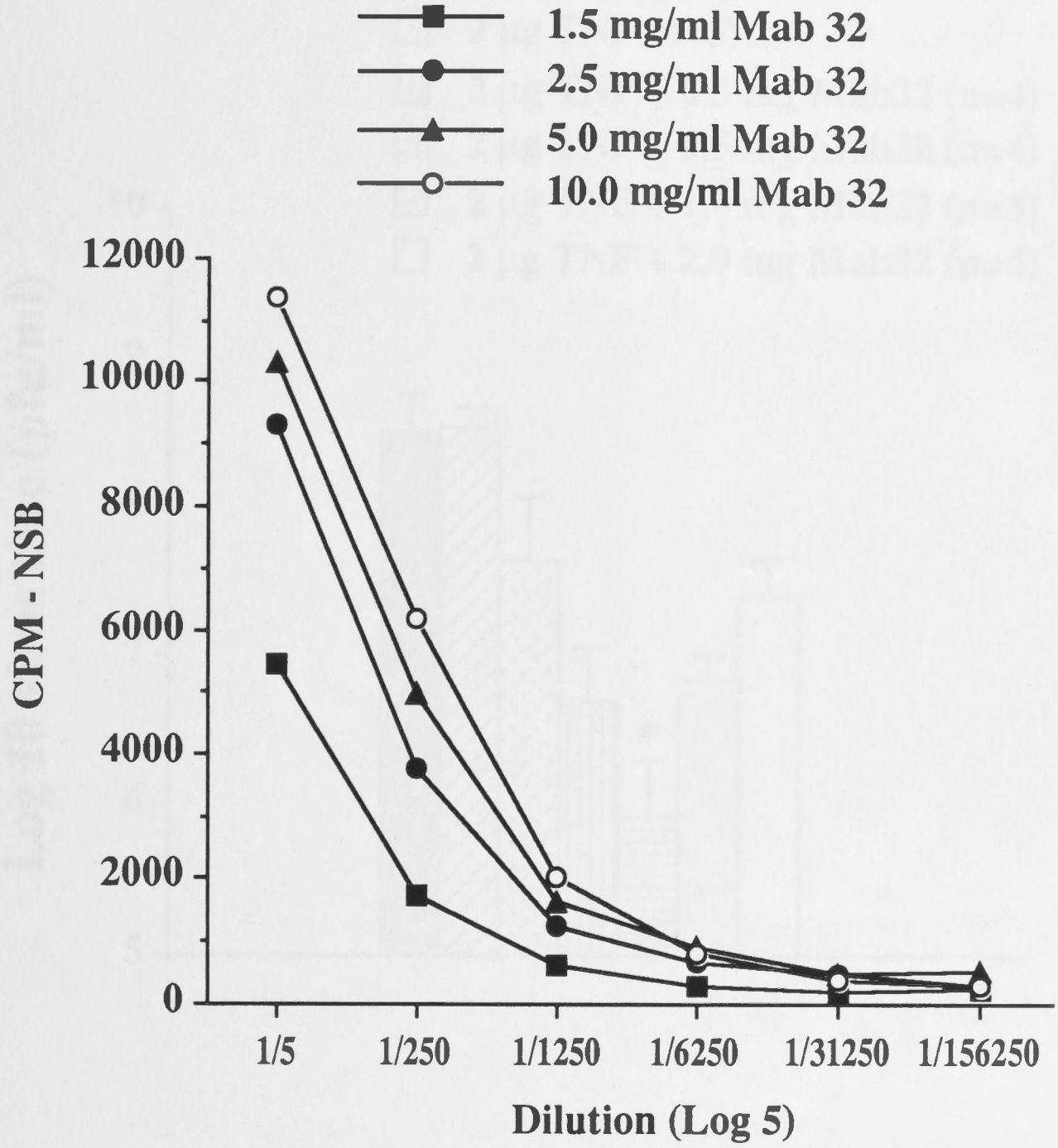


Figure 3.1 The binding of ^{125}I-TNF to varying doses of Mab 32 in a radioimmunoassay using SAC cells.

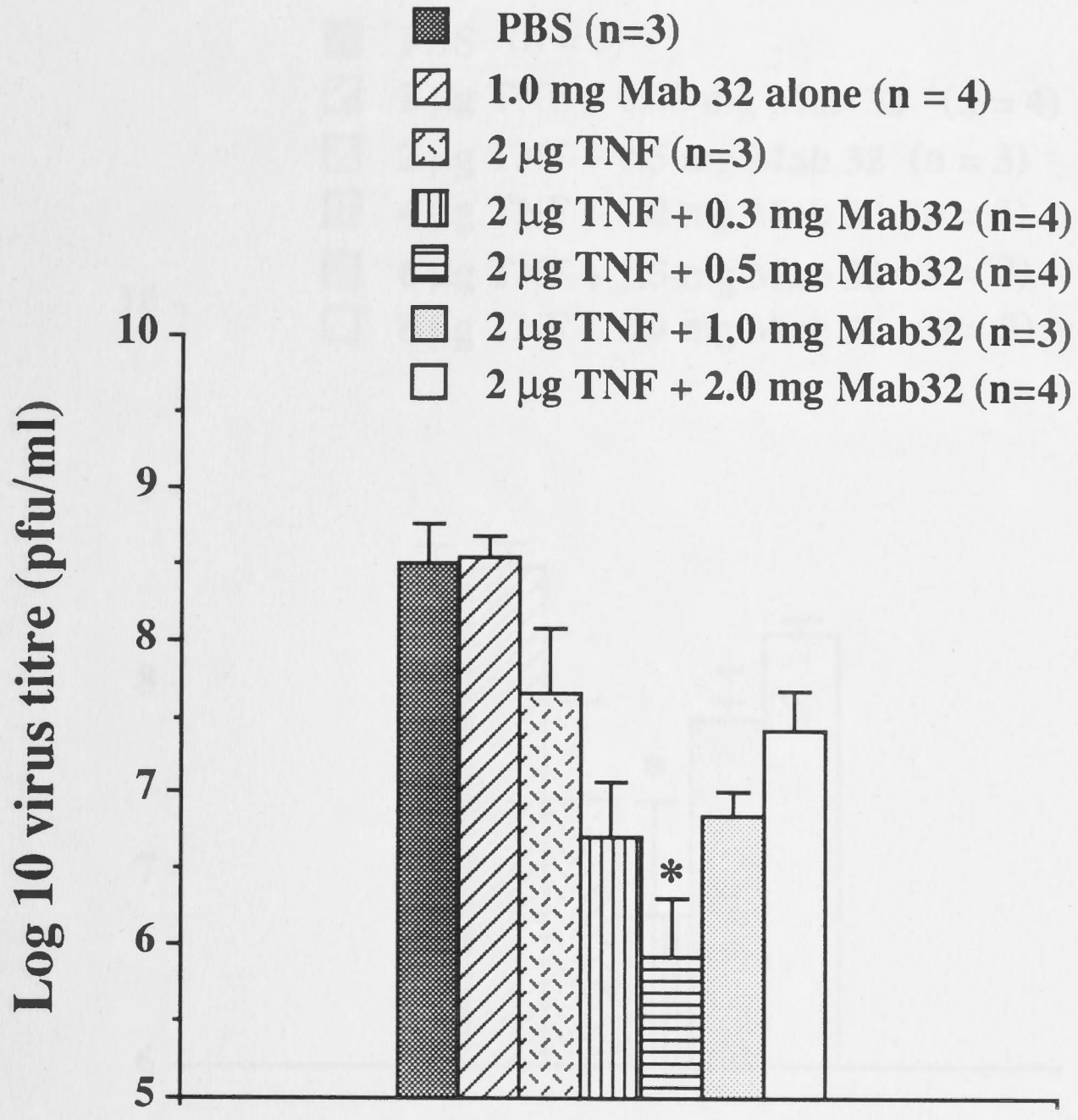


Figure 3.2 Mean growth of vaccinia virus in the ovaries of CBA/H mice treated 24 hours before infection with either TNF alone, Mab 32 alone or TNF in complex with Mab 32 at the indicated doses.

* p = 0.029 versus TNF alone.

The TNF alone treatment was not significantly different from the PBS control (p = 0.163).

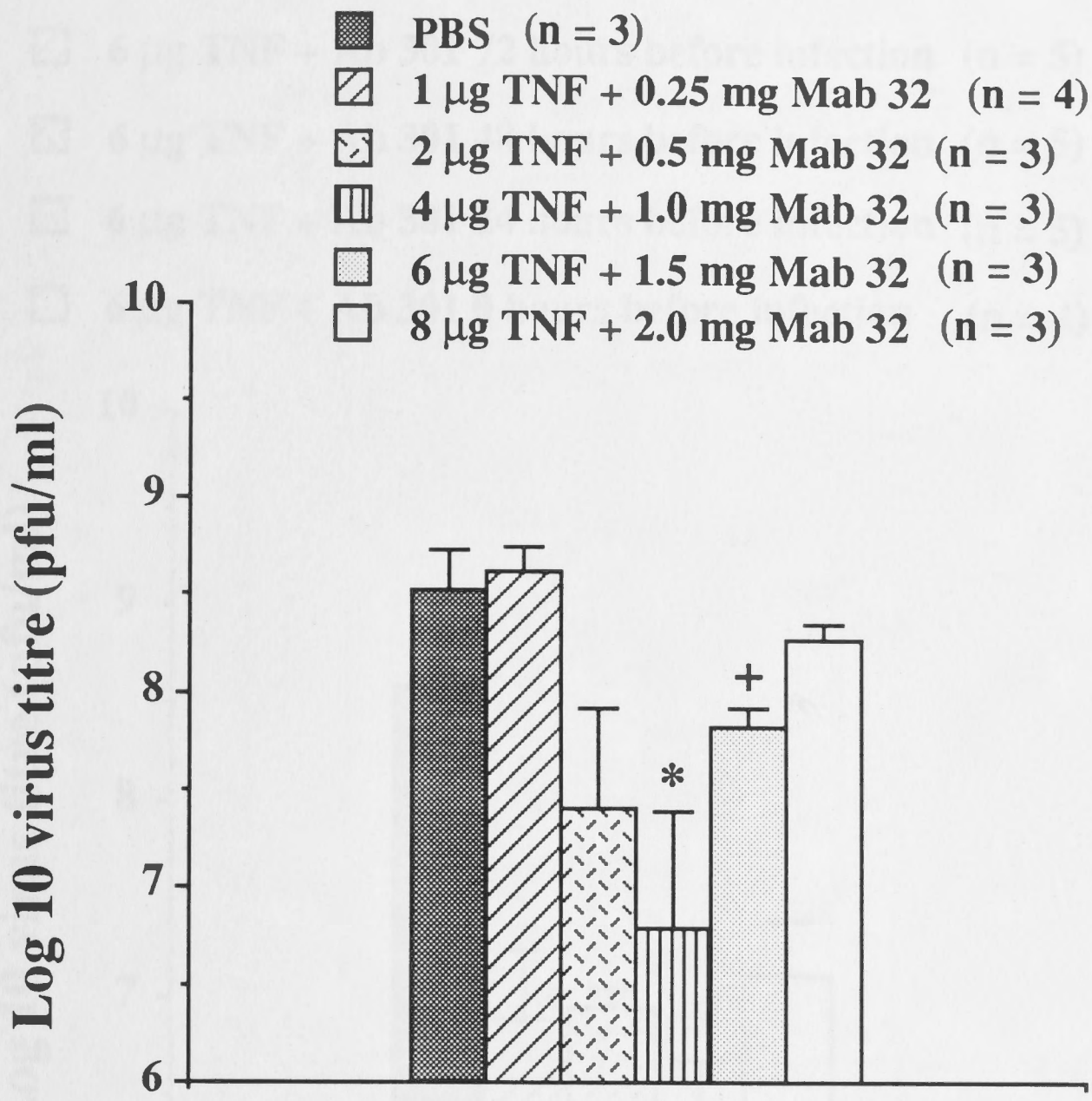


Figure 3.3 Mean growth of vaccinia virus in the ovaries of CBA/H mice treated 24 hours before infection with increasing doses of TNF in complex with increasing concentrations of Mab 32, following the ratio of 1 μg TNF : 0.25 mg Mab 32.

* p = 0.08 versus PBS treatment.

+ p = 0.05 versus PBS treatment.

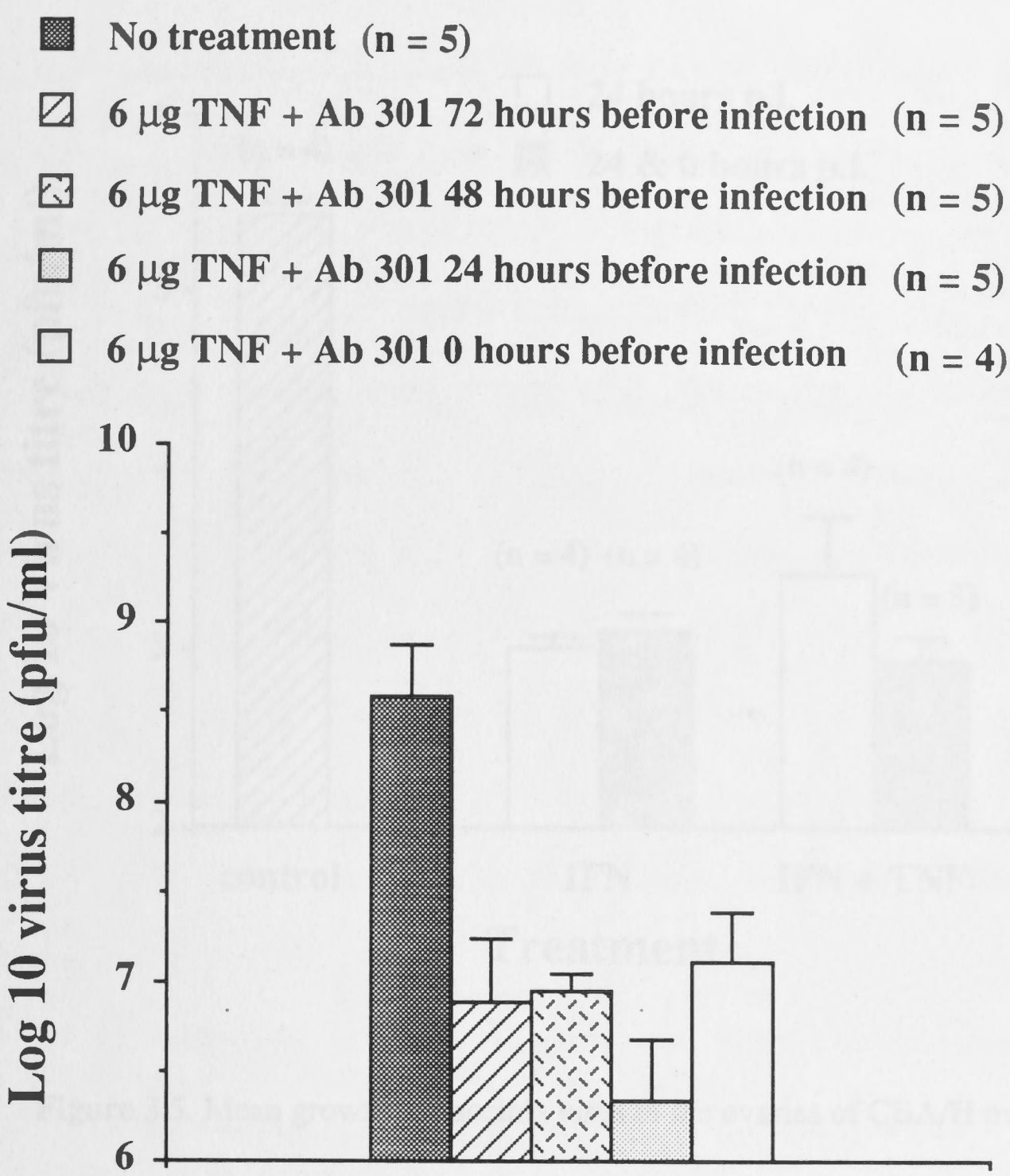


Figure 3.4 Mean growth of vaccinia virus in the ovaries of CBA/H mice treated with 6 μg TNF plus 1/50 Ab 301 at 72, 48, 24 and 0 hours before infection.

All TNF + Ab 301 therapies had significantly lower virus titres compared to the PBS control ($0.01 > p \geq 0.001$).

The virus titres for the TNF + Ab 301 therapies did not differ significantly ($0.35 > p > 0.10$).

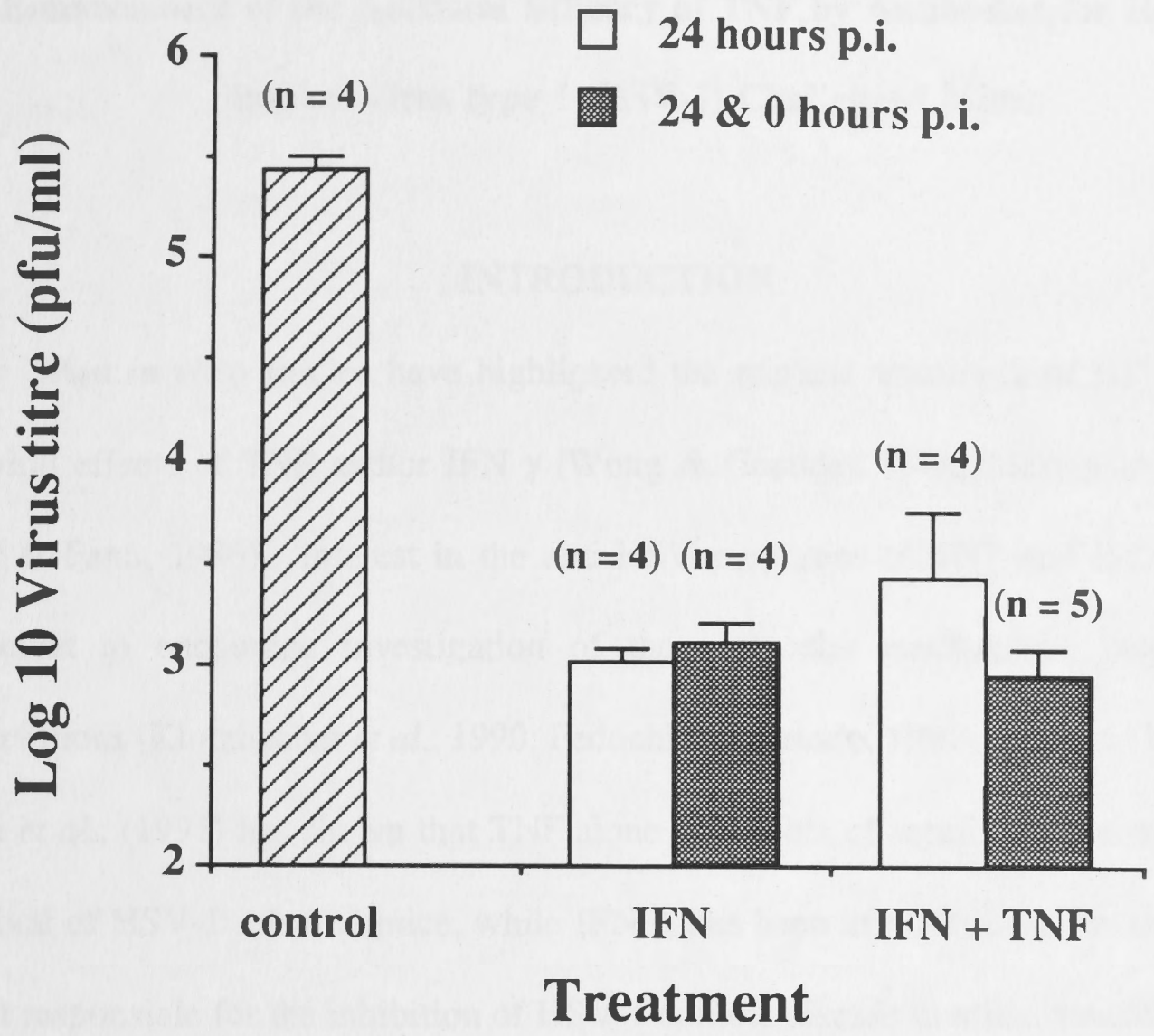


Figure 3.5 Mean growth of vaccinia virus in the ovaries of CBA/H mice treated with IFN γ either alone or in combination with TNF at 24 hours or 24 and 0 hours before infection. The ovaries were removed for titration at day 2 post infection.

All IFN ± TNF therapies resulted in significantly lower titres compared to the PBS control ($p < 0.01$).

There were no significant differences between the titres for the IFN ± TNF treatments ($p > 0.10$).

Chapter 4.

Enhancement of the Antiviral Efficacy of TNF, by Antibodies, for Herpes Simplex Virus type 1 (HSV-1) Challenged Mice.

INTRODUCTION.

Past *in vitro* studies have highlighted the marked sensitivity of HSV-1 to the antiviral effects of TNF and/or IFN γ (Wong & Goeddel, 1986; Mestan *et al.*, 1986; Koff & Fann, 1986). Interest in the anti-HSV properties of TNF and IFN has been sufficient to encourage investigation of the molecular mechanisms behind these observations (Klotzbucher *et al.*, 1990; Feduchi & Carrasco, 1991). A report by Rossol-Voth *et al.*, (1991) has shown that TNF alone is capable of significantly extending the survival of HSV-1 infected mice, while IFN α has been implicated as the endogenous agent responsible for the inhibition of HSV-1 corneal disease in mice (Hendricks *et al.*, 1991). These results demonstrate the potential of particular cytokines to be effective against HSV infection.

As described in the preceding chapter the antiviral efficacy of TNF against vaccinia virus in mice could be enhanced by specific anti-TNF antibodies. Whether this finding was a general phenomenon in virus infection or restricted to only certain virus types needed investigation. This chapter examines the enhancement of TNF by antibodies in the context of HSV-1 infection.

MATERIALS and METHODS.

Treatment of mice with cytokines and infection with HSV-1.

Six micrograms of hTNF was mixed with a 1/50 dilution of Ab 301 and allowed to stand for 1 hour at room temperature. This TNF/Ab 301 ratio was the most effective in the vaccinia virus models. After the incubation period this stock solution was diluted according to the required TNF concentration (ie, 0.25, 0.5, 1.0 or 2.0 μg/mouse) in sterile PBS. TNF and Ab 301 were therefore at the same ratio across the TNF dose range used. TNF without Ab 301 was also made up in sterile PBS at the concentrations described. These TNF treatments were subsequently administered i.p. to CBA/H mice.

In some experiments CBA/H mice received 10^5 U mIFN γ alone or a combination treatment of 2 μg TNF and 10^5 U mIFN γ again administered i.p. BALB/c and C57/B6 mice only received the 2 μg TNF alone, 10^5 U mIFN-γ alone or a combination of the TNF and IFN treatments i.p. Twenty-four hours after cytokine treatment the mice were infected i.p. with 10^7 pfu of HSV-1. Three days post infection the relevant organs were removed for titration.

N.B. In the experiment described in Fig. 4.4 the mice were first infected i.p. with 10^7 pfu of HSV-1 and at 4 and 24 hours post infection treated i.p. with 2 μg TNF alone or 2 μg TNF plus Ab 301.

Measurement of virus growth *in vivo*.

As with vaccinia virus the growth of HSV-1 was determined via measuring virus growth in particular organs. HSV-1 also grew to comparatively higher titres in the ovaries, hence the choice of this organ in these experiments. HSV-1 was found to grow to low titres in the spleen and liver. The adrenal gland has been described as a model

organ for HSV-1 studies previously (Hill *et al.*, 1986; Potratz *et al.*, 1986).

Three days post infection (p.i.) the mice were sacrificed by cervical dislocation and the ovaries and/or adrenal glands aseptically removed. These organs were subsequently homogenised in 1.0 ml of sterile PBS afterwhich 100 µl of homogenate was incubated at 37^{0}C for 30 minutes in 0.1% trypsin. Trypsinisation was stopped by the addition of foetal calf serum (FCS; Cytosystems, Sydney). These homogenates were 10-fold serially diluted in sterile PBS and 100 µl of these dilutions plated out on a confluent monolayer of Vero cells that had been seeded into Linbro flat-bottomed 6 well plates at a concentration of 10^6 cells / well the previous day in RPMI or MEM (F15) plus 5 % heat-inactivated FCS. The plates containing the virus dilutions were left at 37^{0}C (5% CO_2, humidified atmosphere) for 1 hour before being overlaid with 2.5 mls of medium. The plates were then incubated for 2 days at 37^{0}C afterwhich the medium was aspirated and the cell monolayers stained with 0.1 % (w/v) crystal violet in 20 % (v/v) ethanol. The plates were air-dried, the plaques were counted and the mean (±SEM) concentration of virus (Log_{10} virus [pfu/ml]) calculated. Statistical significance was calculated using an unpaired Student's t-test. As with the vaccinia virus titrations described previously, this assay is limited to the measurement of virus titres greater than 2.0 logs; i.e., 1 plaque at the 10^{-1} dilution. Samples with no detectable virus were counted as having 1.8 logs of virus.

* The experiments performed for this chapter were all done at least twice unless otherwise indicated. Please note that (n =) refers to the number of animals or samples in an experimental or control group.

RESULTS.

Ab 301 does not enhance the antiviral effect of TNF at doses of 2 or 6 μg in the adrenal glands or ovaries of HSV-1 challenged mice.

TNF concentrations which were enhanced by Mab 32 and Ab 301 in the previously described vaccinia virus model were tried for activity against HSV-1 in CBA/H mice. In both the adrenal glands (Fig. 4.1 a) and ovaries (Fig. 4.1 b) no enhancement of antiviral effect was seen when TNF was used at either dose in the presence of Ab 301. The TNF treatments alone however did have antiviral activity as opposed to the findings with vaccinia virus. In the adrenal glands both doses of TNF alone resulted in significant decreases in HSV-1 titre compared to the PBS treated controls. In the ovaries only the 2 μg TNF treatment showed a significant reduction in HSV-1 titre compared to the PBS treated controls. In both organs tested there was no significant difference in virus titre between the two TNF alone doses.

The enhancement of TNF doses less than 2.0 μg by Ab 301.

Figure 4.2 a) shows that at the doses of 1.0 and 2.0 μg TNF alone there was a significant decrease in the HSV-1 titre compared to PBS treated controls. At neither of these doses was an enhancement of this antiviral effect seen with Ab 301. The TNF dose of 0.5 μg did not result in a significant decrease in HSV-1 titre in comparison with the PBS control. This dose in complex with Ab 301 did, however, display a significant reduction of HSV-1 titre in comparison with the PBS treated controls. When 0.5 μg TNF plus Ab 301 was compared to the 0.5 μg TNF alone treatment a conspicious decrease in HSV-1 titre was also evident (Fig. 4.2 a,b). At the 0.25 μg TNF concentration (Fig. 4.2 b) there was no significant decrease in HSV-1 titre either with or without Ab 301 compared to PBS treatment nor was there an enhancement of the

antiviral efficacy of this TNF dose in the presence of Ab 301 (Fig. 4.2 b).

The effects of TNF and mIFN γ co-administration in HSV-1 challenged mice.

Unlike vaccinia virus HSV-1 is responsive to prior administration of TNF, at certain doses, without Ab 301. Experiments were therefore performed with 2 μg of TNF and 10^5 U mIFN γ in CBA/H mice to look for synergy in the antiviral effects of these cytokines. It was also of interest to see whether TNF and IFN either alone or together were effective in BALB/c (HSV susceptible) and C57/B6 (HSV resistant) mice.

Figure 4.3 shows the results of an experiment where TNF and IFN were administered to CBA/H mice. As demonstrated earlier the 2 μg TNF alone dose resulted in a significant decrease in HSV-1 titre in comparison to the PBS treatment. The IFN alone treatment also resulted in a significant decrease in HSV-1 titre compared to the control treatment. The titres for the TNF alone and IFN alone therapies did not significantly differ. When TNF and IFN were co-administered there was a significant decrease in HSV-1 titre in comparison to the titres found for the individual cytokine therapies.

The administration of 2 μg of TNF alone 24 hours before infection to C57/B6 mice had a dramatic effect on the growth of HSV-1 (Table 4.1). The mean virus titre was reduced to below detectable limits (< 2.0 $\log_{10}$). PBS treated mice had mean HSV-1 titre of 4.78 $\log_{10}$. The doses of 10^5 U mIFN-γ alone and together with TNF also showed dramatic reductions in the growth of HSV-1. The TNF plus IFN therapy was not significantly different to the IFN alone therapy. These findings highlight the sensitivity of a HSV resistant mouse strain, C57/B6, to exogenous treatment with TNF and/or IFN. Table 4.1 also shows the results of the same experiment done with BALB/c mice. Although HSV-1 grew in PBS treated BALB/c mice to a titre similar

to that for PBS treated C57/B6 mice the response of this strain to HSV-1 growth restriction by TNF and IFN either individually or together was not significant. These results may delineate important mechanisms in the relative resistance/susceptibility of these 2 mouse strains to HSV-1 infections.

TABLE 4.1 Mean growth of HSV-1 (Log_{10} virus titre [pfu/ml] ± SEM) in the ovaries of cytokine pre-treated C57/B6 and BALB/c mice.

Treatment	C57/B6	BALB/c
PBS	4.78 ± 0.19	4.24 ± 0.52
2 μg TNF	< 2.0 *	4.15 ± 0.10 +
10^5 U mIFN-γ	2.43 ± 0.35 *	3.64 ± 0.18 +
2 μg TNF + 10^5 U mIFN-γ	2.23 ± 0.26 *	3.92 ± 0.18 +

* $p < 0.001$ versus C57/B6 PBS treatment.

+ $0.89 > p > 0.27$ versus BALB/c PBS treatment.

The clearance of HSV-1 by the administration of TNF alone or TNF in complex with Ab 301 at 4 and 24 hours post-infection.

It has been noted in the past that TNF is able to selectively lyse HSV-1 infected cells *in vitro* (Koff & Fann, 1986). An experiment was therefore performed to address the possibility of this selective targeting occurring *in vivo* as well as observing the influence of Ab 301 on this effect.

There was no significant difference in HSV-1 growth between the PBS treated

controls and the 4 hour or 24 hour post infection (p.i.) therapies either with or without Ab 301 (Fig. 4.4). There were some interesting effects seen between the TNF alone and TNF plus Ab 301 therapies. At the 4 hour p.i. time point there was an increase in the HSV-1 titre resulting from the TNF plus Ab 301 therapy compared to the TNF alone therapy. At the 24 hour p.i. time point the mean HSV-1 titre for TNF plus Ab 301 was lower in comparison to the TNF alone treatment but this difference was not significant. The HSV-1 titre however was significantly lower at the 24 hour p.i. time point for the TNF plus Ab 301 treated mice compared to the titre found in similarly treated mice at 4 hours p.i. There was no significant difference found between the TNF alone treatments at the 4 and 24 hour p.i. time points.

It appears that the TNF plus Ab 301 complex is more active in this HSV-1 pre-infection model than TNF alone. The results also suggest that HSV-1 growth can vary on exposure to TNF according to the time that the infection has been in progress.

SUMMARY.

This chapter examined the enhancement of TNF antiviral effect by antibodies in HSV-1 infections. Using an *in vivo* infection model similar to that used for vaccinia virus, it was found that the response of HSV-1 to TNF both with and without Ab 301 was very different to that found for vaccinia virus. TNF alone treatments were able to significantly inhibit the *in vivo* growth of HSV-1, and no significant enhancement of antiviral effects were seen when TNF was in complex with Ab 301. Studies of TNF alone with IFN γ showed an additive reduction in HSV-1 growth in CBA/H mice. It did appear, however, that the magnitude of antiviral response was dependent upon the genetic background of the mouse. This was highlighted in the comparison of TNF and/or IFN γ responses in C57/B6 (HSV resistant) versus BALB/c (HSV sensitive) mice. Dramatic reductions in virus growth were seen in B6 mice for each cytokine therapy tested (particularly TNF alone), whereas BALB/c mice showed little antiviral response when treated with the same cytokines. It also appears that the timing of exogenous TNF exposure to mice already infected with HSV-1 can be a factor in HSV-1 growth.

The results of this chapter emphasise that the enhancement of TNF antiviral efficacy by antibodies is not a general property to be applied to all virus families.

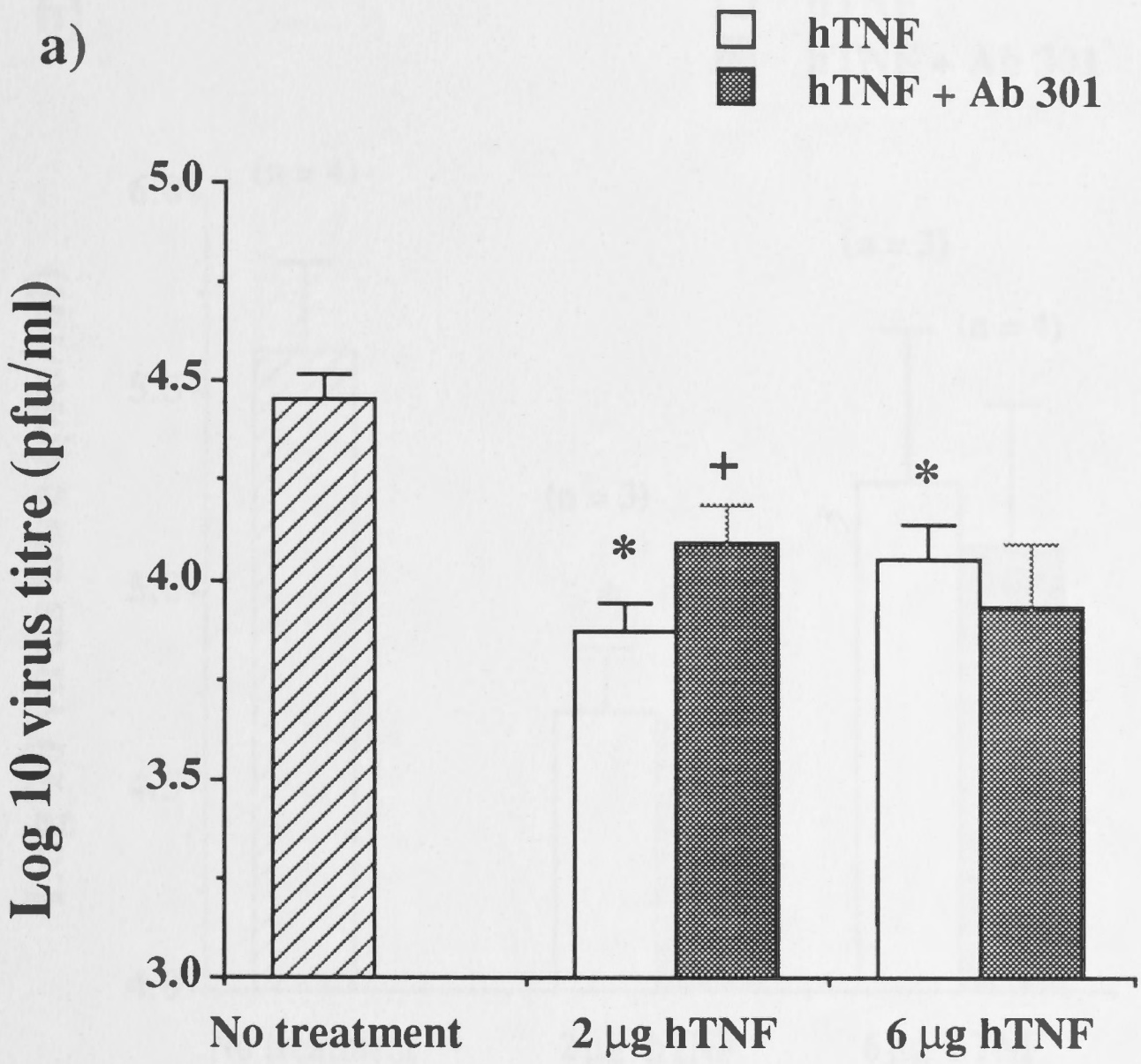


Figure 4.1 a) Mean growth of HSV-1 in the adrenal glands of CBA/H mice pre-treated with 2 or 6 μg TNF ± Ab 301 24 hours before infection. **(n = 4)** for each treatment.

* $p < 0.02$ versus PBS treatment .

+ Not significantly different to the TNF alone therapy.

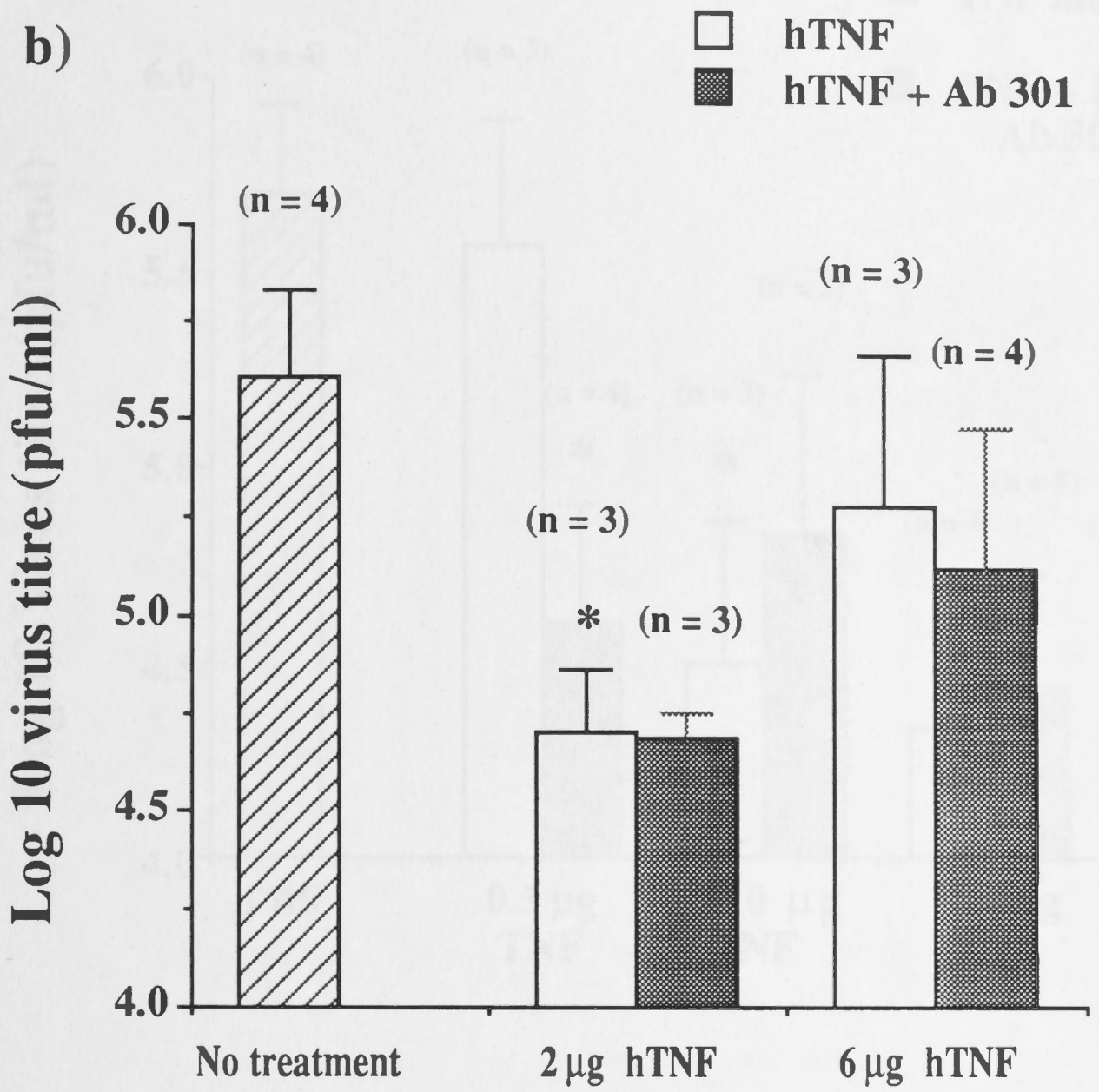


Figure 4.1 b) Mean growth of HSV-1 in the ovaries of CBA/H mice pretreated with 2 or 6 μg TNF ± Ab 301 24 hours before infection.

* p = 0.025 versus PBS treatment.

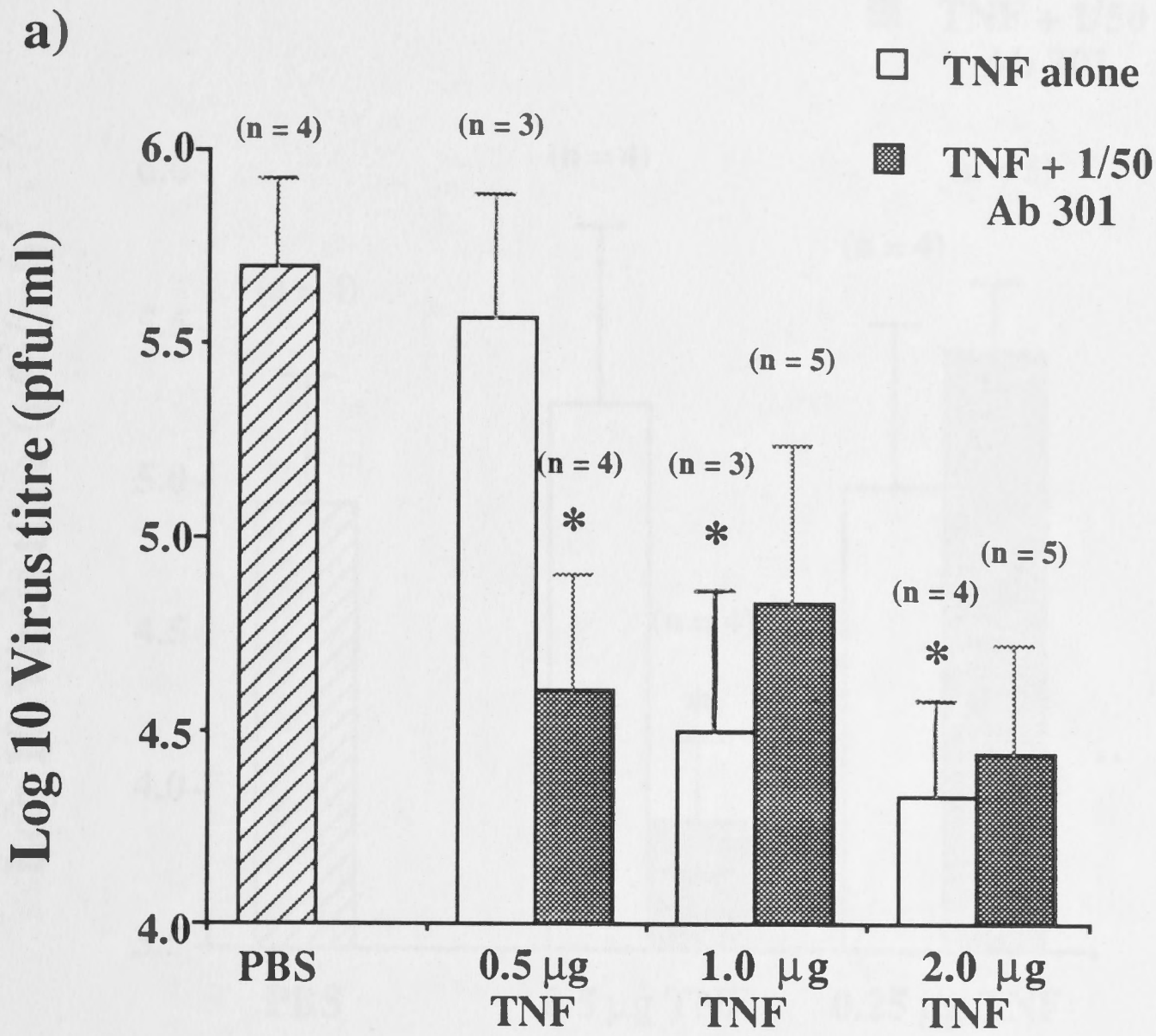


Figure 4.2 a) Mean growth of HSV-1 in the ovaries of CBA/H mice pretreated with 0.5, 1.0 or 2.0 µg TNF ± Ab 301 24 hours before infection.

* $0.035 > p > 0.005$ versus PBS treatment.

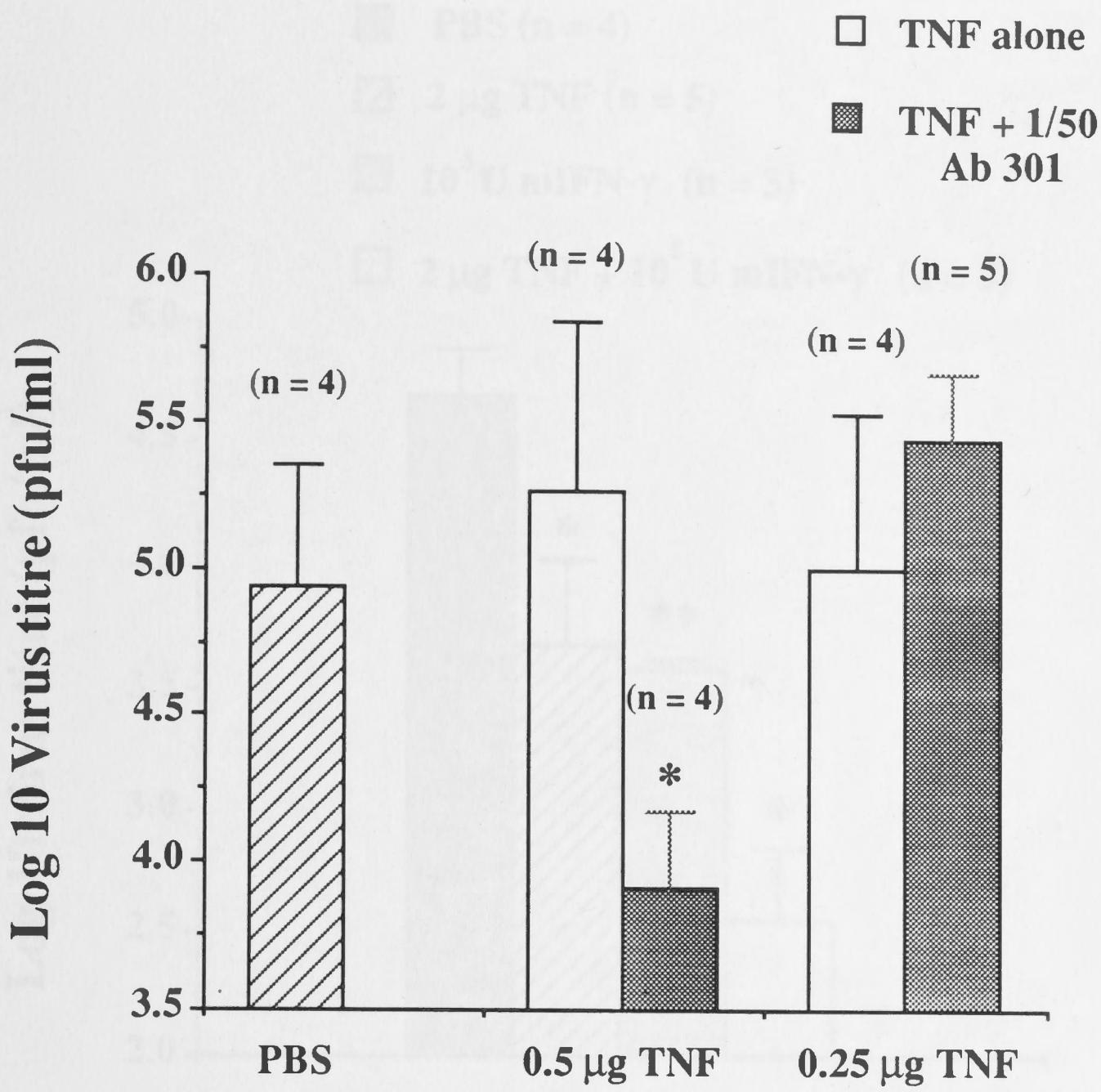


Figure 4.2 b) Mean growth of HSV-1 in the ovaries of CBA/H mice pretreated with 0.5 or 0.25 µg TNF ± Ab 301 24 hours before infection.

* p = 0.079 versus 0.5 µg TNF alone treatment.

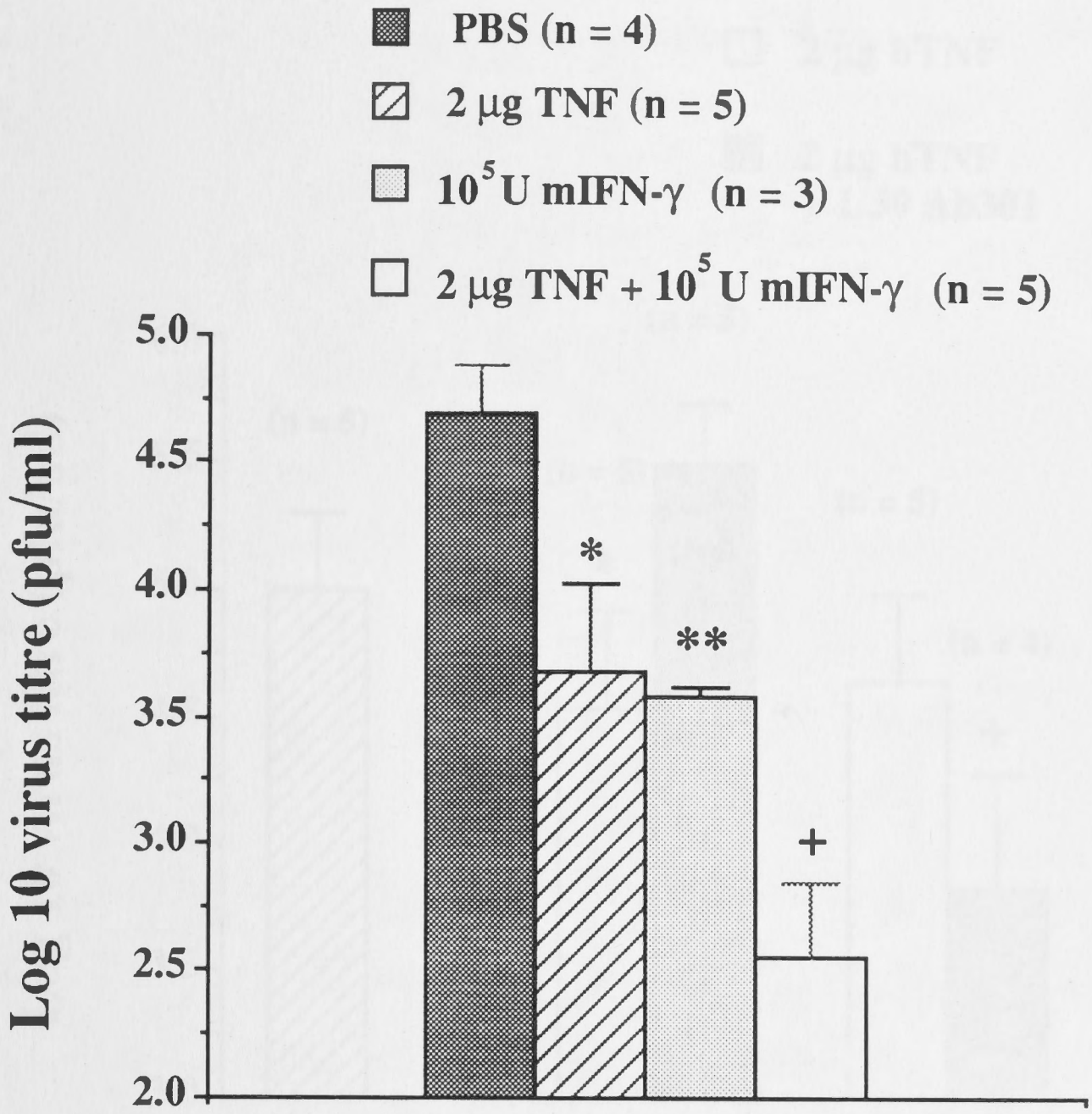


Figure 4.3 Mean growth of HSV-1 in the ovaries of CBA/H mice pretreated with 2 μg TNF and 10^5 U mIFN γ either alone or together 24 hours prior to HSV-1 infection.

* p = 0.046 versus PBS treatment.

** p = 0.005 versus PBS treatment.

\+ Significantly different to the TNF alone (p = 0.035) and IFN alone (p = 0.038) therapies.

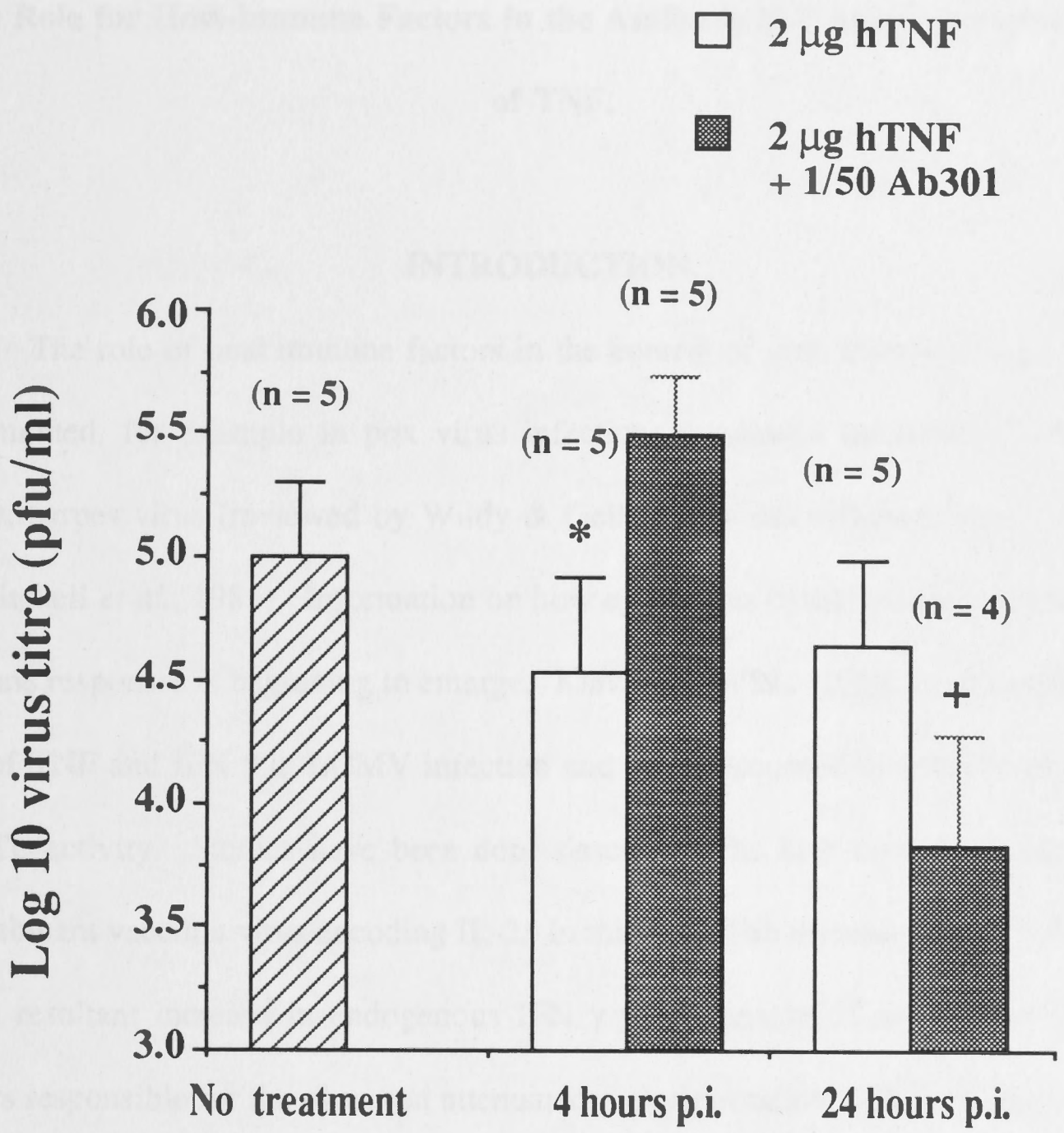


Figure 4.4 Mean growth of HSV-1 in the ovaries of CBA/H mice that had been first infected (10^7 pfu, i.p.) and at 4 and 24 hours post infection treated with 2 μg TNF ± Ab 301.

* p = 0.07 versus TNF plus Ab 301 at 4 hours post infection.

+ p = 0.011 versus TNF plus Ab 301 at 4 hours post infection.

*The above described data were the results of a single experiment.

Chapter 5.

The Role for Host-Immune Factors in the Antibody Enhanced Antiviral Effect of TNF.

INTRODUCTION.

The role of host immune factors in the control of viral infection has been well documented, for example in pox virus infections (reviewed by Buller & Palumbo, 1991), herpes virus (reviewed by Wildy & Gell, 1985) and influenza virus (reviewed by Mitchell *et al.*, 1985). Information on how exogenous cytokines may augment host immune responses is beginning to emerge. Klavinskis *et al.*, (1989) have examined the role of TNF and IFN γ in LCMV infection and have discussed their findings in terms of CTL activity. Studies have been done describing the host immune response to a recombinant vaccinia virus encoding IL-2. In this model an increase in NK cell activity and a resultant increase in endogenous IFN γ were considered to be the major host factors responsible for the observed attenuated growth of this VV-IL-2 (Karupiah *et al.*, 1990a, 1990b). Similarly constructed recombinant vaccinia viruses encoding TNF (Sambhi *et al.*, 1991) or mIFN γ (Kohonen-Corish *et al.*, 1990) have also been shown to have attenuated growth *in vivo*, but contributing host factors have not been clearly defined.

This chapter examines the role of host immune factors in the observed antibody enhanced antiviral effects of TNF in a vaccinia virus infection. As well as considering immune cell activity in this phenomena, some attention will also be given to the possible induction of non-specific effector molecules in mice that had been pre-treated with TNF ± Ab 301 and subsequently infected with vaccinia virus.

MATERIALS and METHODS.

Irradiation of mice.

CBA/H female mice were sub-lethally gamma-irradiated (650 Rads) by a Cobalt-60 (^{60}Co) source in order to deplete their proliferative cellular immune response. Twenty-four hours post-irradiation animals were treated with cytokines and 24 hours later the mice were infected with 10^6 pfu vaccinia virus i.v.

Natural Killer (NK) Cell assay.

i) Preparation of Spleen (effector) cells.

Groups of CBA/H female mice (n = 3 per treatment) were treated i.p. with one of the following regimes: PBS, 6 μg hTNF alone, 6 μg hTNF plus 1/50 Ab 301, 10^5 U mIFN γ or a combination of TNF, Ab 301 and mIFN at the doses indicated (this treatment was performed exactly as described in chapter 3). Twenty-four hours later these cytokine treated mice were challenged with 10^7 pfu of vaccinia virus i.v. An additional group of 3 mice which were neither cytokine treated nor challenged with vaccinia virus served as controls. Two days post-infection the mice were sacrificed by cervical dislocation and the spleens aseptically removed, collected into DMEM + 10 % FCS and immediately placed on ice. The spleens from each treatment group were pooled. The pooled spleens were passed through a sterile stainless steel mesh with a syringe plunger. Erythrocytes were removed by water lysis (isotonic conditions were returned by adding 10 x HBSS). The cell and tissue debris was allowed to settle, the cell containing supernatants transferred to a clean sterile tube and centrifuged (Beckman, TJ-6) at 1500 RPM for 5 minutes. The remaining lymphocytes were then counted, washed once and resuspended in DMEM plus 10 % FCS at the desired concentration. These cells were then plated in triplicate into Linbro 96-well round-

bottomed plates (100 µl / well) at the indicated ratio to YAC-1 (NK cell sensitive) target cells. Effector : target ratios used were; 2:1, 7:1 and 20:1.

ii) Preparation of YAC-1 target cells.

YAC-1 cells (2 x 10^6) were labelled with sodium chromate (^{51}Cr: Amersham, U.K.) in a volume of 200 µl DMEM plus 10 % FCS for 1.5 hours at 37^{0}C. A dose of 50 µCi was used per 2 x 10^6 cells. After the 1.5 hour labelling period the YAC-1 cells were washed 3 x in DMEM plus 10 % FCS. The labelled cells were diluted accordingly in DMEM plus 10 % FCS and placed into a round-bottomed 96-well plate (containing the spleen lymphocytes) at a concentration of 2.0 x 10^4 cells / well in 100 µl.

iii) Estimation of Specific lysis.

The plates were incubated for 4 hours at 37^{0}C in a humidified atmosphere containing 5 % CO_2. NK cell activity was measured as the amount of radioactivity released from the ^{51}Cr labelled YAC-1 cells into the assay supernatants and counted (as CPM) on a Packard Auto-Gamma counter. The specific lysis of the NK cell sensitive YAC-1 target cells was calculated by the following formula:

$$\% \text{ Specific Lysis} = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximal release - Spontaneous release}} \times 100.$$

Spontaneous release was measured as the amount of radioactivity in the supernatants of ^{51}Cr labelled YAC-1 cells incubated under assay conditions but without spleen cells. Maximum release was the radioactivity released into the supernatants of ^{51}Cr labelled YAC-1 cells incubated in 1.0 % Triton-X detergent. Spontaneous release ranged between 10-15 % for YAC-1 cells.

Cytotoxic T-cell (CTL) assay.

i) Preparation of Spleen (effector) cells.

Groups of 3 female CBA/H mice were administered one of the following treatments i.p.: PBS, 6 μg TNF or 6 μg TNF + 1/50 Ab 301. Twenty-four hours later these mice were challenged with 10^7 pfu vaccinia virus (VV-WR-L929) i.v. An additional group of 3 mice which were neither treated with cytokines nor infected served as controls. Six days p.i. the mice were sacrificed by cervical dislocation and the spleens removed and processed as described for the NK cell assay (except that RPMI plus 5 % FCS was used). Effector : target ratios used were; 2:1, 7:1, 20:1 and 60:1.

ii) Preparation of L929 (target) cells.

L929 cells (2 x 10^6) were incubated with ^{51}Cr (50 μCi / 2 x 10^6 cells) in a volume of 200 μl of RPMI plus 5 % FCS for 1.5 hours at 37^{0}C in the presence of VV-WR-L929 (MOI = 20). Another set of L929 cells were also labelled with ^{51}Cr at the same dose but not infected with VV-WR-L929. These cells were controls to gauge the degree of non-specific lysis of targets by spleen cells from infected mice. After the labelling/infection period the cells were washed 3 x in RPMI plus 5 % FCS, diluted accordingly in RPMI plus 5 % FCS and plated out into round-bottomed 96 well plates (containing the effector cells) at a concentration of 2.0 x 10^4 cells / well.

iii) Estimation of Specific lysis.

The plates were incubated for 6 hours at 37^{0}C in a humidified atmosphere containing 5 % CO_2. After this incubation period the supernatants were collected and processed exactly the same way as described for NK cells (see above). Spontaneous

release for L929 cells (infected and non-infected) ranged between 10-15 %.

Differentiation of resident peritoneal cells in TNF ± Ab 301 treated and vaccinia infected mice.

Groups of 3 female CBA/H mice were injected i.p. with either PBS, 6 μg TNF or 6 μg TNF + Ab 301. Twenty-four hours later these mice were challenged with 10^7 pfu vaccinia virus i.v. A control group of 3 mice were left untreated and uninfected. At day 1 p.i. the mice were sacrificed and their peritoneal cavities lavaged with 1.0 ml of sterile and ice-cold PBS. The peritoneal washings were then microfuged (Eppendorf) for 5 minutes at 1500 RPM. The supernatants were U.V. irradiated (10 cm for 5 minutes), transferred to sterile eppendorf tubes and kept for an antiviral assay and RNI estimation (see below). The cell pellet was resuspended in 10 μl of sterile PBS, vortexed and a smear made on clean glass slides. The smears were air-dried, fixed and stained using the Diff-Quick (Lab-Aids, Narrabeen, NSW, Australia) method. It must be noted that the manufacturers warn that this method does not reliably stain eosinophils and basophils. The stained slides were subsequently examined under oil-immersion and the percentage macrophage/monocytes, lymphocytes and polymorphonuclear cells determined.

Antiviral activity of peritoneal lavage supernatants.

The U.V. irradiated supernatants of the above resident peritoneal macrophages were examined for their ability to induce an antiviral state in L929 cells.

One hundred microlitres of these supernatants were placed into individual wells of Linbro 24 well plates. One millilitre of RPMI plus 5 % FCS containing 5.0×10^5 L929 cells were then added to the supernatant containing wells. These plates were then

incubated at 37^{0}C in a humidified atmosphere containing 5 % CO_2 for 24 hours. When this 24 hour period had elapsed the medium was removed and the cell monolayers infected with HSV-1* (MOI = 1.0) in 100 µl of PBS for 1.0 hour at 37^{0}C. After the 1.0 hour absorption period the virus plus PBS was removed and fresh RPMI plus 5 % FCS added to each well. The plates were incubated for 48 hours at 37^{0}C after which the plates were frozen and thawed twice. The cell lysates were titrated (10 fold serial dilutions) in PBS and were plated out on Vero cells for a standard HSV-1 plaque assay (described in chapter 4). The reduction in the number of plaques compared to a group of control mice was taken to indicate antiviral activity in the resident peritoneal macrophage lavage supernatants.

* Vesicular stomatitis virus (VSV) is often used as an indicator virus for interferon bio-activity. VSV is not available in Australia. In earlier studies it was found that HSV-1 was exquisitively sensitive to mIFN γ, especially at HSV-1 concentrations of MOI 1.0 - 2.0.

Detection of RNIs.

Reactive nitrogen intermediates were detected in the peritoneal lavage supernatants by an assay which utilises a copper-cadmium-zinc catalyst to convert nitrate to nitrite before exposure to Greiss reagent. This technique has been previously described (Rockett *et al.*, 1992). The standards were diluted in PBS.

Detection of exogenous human TNF ± Mab 32/Ab 301 in the murine peritoneal cavity.

i) Labelling of specific hTNF antibody probes.

Specific hTNF monoclonal antibodies (Mab 11 and Mab 42; both IgG1) derived from a panel of monoclonals mapped and described by Rathjen *et al.* (1991) were biotinylated according to the method described by Hudson and Hay (1989). Briefly, the antibodies were equilibrated in 0.1 M sodium bicarbonate buffer (pH 8.4) by dialysis. The concentration of the antibodies was adjusted to 1.0 mg/ml in bicarbonate. The required amount of biotin succinimide ester (Sigma, St. Louis) was weighed out and dissolved in dimethyl sulphoxide at a concentration of 1.0 mg/ml immediately before use. For every 1.0 mg of protein 120 µl of biotin succinimide ester in DMSO was added and the solution mixed immediately. This mixture was incubated at room temperature with agitation for 4 hours. The conjugate solution was finally dialysed overnight against PBS plus 0.02 % azide.

ii) Sandwich ELISA assay for the detection of hTNF.

Nunc maxisorp plates were coated with a cocktail of monoclonal antibodies (Mabs 1, 37, and 54; all IgG1) specific to a hTNF domain that did not overlap with the domain to which the above described biotinylated Mabs would bind. These capture antibodies were from the same panel as described above (Rathjen *et al.*, 1991). The capture antibodies (20 µg/ml) were coated onto the plates in 0.05 M carbonate buffer, pH 9.6, and left overnight at 4^0C. The plates were blocked by the addition of 1.0 % BSA in PBS and left for 60 minutes at 37^0C. The samples were then added and the plates incubated at 37^0C for 60 minutes. After this incubation the biotinylated probe antibodies were added at a concentration of 10 µg/ml to the plates after which the plates

were incubated at 4^0C overnight. Both the samples and the biotinylated Mabs were diluted in PBS plus 1 % BSA. At the completion of the overnight incubation a streptavidin-peroxidase conjugate was made using an AV-ABC kit (Vectstain, Vector, Calif., USA) with the manufacturers directions being followed to make this conjugate. The conjugate was added to the plates for 1 hour at 37^0C. The plates were washed six times between each of the above steps with PBS containing 0.05 % Tween 20. Finally, the peroxidase substrate was added. This substrate consisted of 10 mg 1,2-phenylene diamine (Sigma, St. Louis) and 9.4 µl 30 % hydrogen peroxide per 12.5 mls 0.3 M citrate/phosphate buffer. The plates were then left at room temperature for 30 minutes in the dark, after which 50 µl of 2.5 M sulphuric acid (H_2SO_4) was added to each well. The plates were then read immediately at 490 nm (690 nm reference) on a Dynatech plate reader. The unknown values were calculated from a hTNF standard curve (3rd order polynomial transformed). Murine TNF could not be detected using this ELISA method (see Fig. 5.1). The data is represented as mean ± SEM. The binding of TNF alone in relation to TNF + Mab 32 or TNF + Ab 301 is also described in Fig. 5.1.

* The experiments performed for this chapter were all done at least twice. The results described in Tables 5.4, 5.5 and 5.6 were obtained from the same groups of CBA/H mice, and are the results of only one experiment. Please note that (n =) refers to the number of animals or samples in an experimental or control group.

RESULTS.

Ab 301 increases the persistence of TNF in the peritoneal cavity of CBA/H mice.

The comparative detection of TNF either alone or plus Mab 32 or Ab 301 is shown in Fig. 5.1. This figure also shows that using this ELISA method murine TNF is not significantly detected. Because the detection of TNF plus Ab 301 had the greatest similarity to that found for TNF alone this antibody was used in the *in vivo* studies. TNF plus Mab 32 registered higher optical density readings, particularly in the lower dilutions, than TNF plus Ab 301 or TNF alone.

Table 5.1 describes the measurement by ELISA of exogenous hTNF either alone or plus Ab 301 in peritoneal lavage samples at certain time points post i.p. treatment. In experiment 1 the amount of TNF detected with or without Ab 301 at 0 hours post treatment did not differ significantly. For both treatments there was a large decrease in TNF concentration by 6 hours post injection. From 6 to 24 hours there were significantly higher concentrations of TNF detected in samples from mice injected with TNF plus Ab 301 compared to samples from TNF alone treated mice. This is particularly so at the 24 hour post-injection time point (ie, the normal time of virus inoculation). A repeat experiment (experiment 2) following this procedure showed again that TNF concentration did not vary significantly at 0 hours post-treatment, but there was significantly more TNF present at 6 hours post-treatment in the samples from TNF plus Ab 301 treated mice. The level of TNF detected in TNF plus Ab 301 treated mice did not significantly vary between the 0 and 6 hour time points. After 24 hours no TNF could be detected in the lavage samples from TNF alone treated mice whereas a trace of TNF was detected in the samples from the mice treated with TNF plus Ab 301. Therefore it appears that the presence of Ab 301 does enhance the persistence of TNF in the peritoneal cavity of CBA/H mice.

TABLE 5.1 The detection, following i.p. injection, of hTNF either alone or in complex with Ab 301 (1/50) in the peritoneal cavity of CBA/H female mice.

	Mean hTNF (ng) ± SEM			
	Experiment 1		Experiment 2	
Hours	6 µg TNF (n=3)	6 µg TNF + Ab301 (n=3)	6 µg TNF (n=3)	6 µg TNF + Ab301 (n=3)
0	167.87 ± 39.27	207.43+ ± 42.77	24.91 ± 5.94	26.73+ ± 8.17
6	15.74 ± 5.13	31.22 (n=1)	3.01 ± 3.01	46.46* ± 13.90
15	18.17 ± 3.01	36.31** ± 2.89	N.D	N.D
24	11.40 ± 2.04	32.36** ± 4.13	0	0.86 ± 0.86

(n = 3) for each treatment unless indicated otherwise. N.D = not done.

+ $p > 0.50$ for TNF alone versus TNF plus Ab 301.

* $p < 0.04$ for TNF alone versus TNF plus Ab 301.

** $p < 0.015$ for TNF alone versus TNF plus Ab 301.

The sensitivity of the antiviral effect induced by TNF plus Ab 301 (± IFN) to sub-lethal irradiation.

To ascertain whether the antiviral effects of TNF plus Ab 301 are mediated by proliferating immune effector cells, for example NK cells, LAK cells and CTLs, mice were exposed to 650 Rads (sub-lethal dose) of gamma irradiation to deplete such

activity. Table 5.2 shows that in CBA/H mice exposed to sub-lethal irradiation there was no loss of antiviral activity either in animals treated with TNF plus Ab 301, IFN γ alone or TNF plus Ab 301 and IFN together (for comparison see tables 3.4 a. and 3.5). This indicates that a proliferating effector cell population such as those described above do not have a role in the antiviral effects induced by these treatments. This is supported by the NK cell and CTL data which follows (Table 5.3 and Fig. 5.2).

TABLE 5.2 Mean growth of vaccinia virus in the ovaries of CBA/H mice which were first exposed to sub-lethal γ irradiation (650 R) followed 24 hours later by cytokine treatment. 10^6 pfu of vaccinia virus was injected i.v 24 hours after cytokine therapy.

Treatment	**Mean $\log_{10}$ virus titre (pfu/ml) ± SEM**
PBS (n = 5)	7.94 ± 0.24
6 μg TNF + 1/50 Ab 301 (n = 5)	4.68 ± 0.67*
10^5 U mIFN γ (n = 5)	6.20 ± 0.37*
6 μg TNF + 1/50 Ab 301 + 10^5 U mIFN γ (n = 4)	2.85 ± 0.98*+

* $p < 0.005$ versus PBS treatment.

\+ $p = 0.010$ versus 10^5 U mIFN γ treatment.

Natural Killer cell activity is not responsible for the TNF plus Ab 301 mediated antiviral effects.

Table 5.3 describes the NK cell activity in the spleens of CBA/H mice pre-treated with cytokines and challenged with 10^7 pfu vaccinia virus (10^6 pfu was found not to elicit very strong NK activity). In mice pre-treated with 6 μg TNF alone there was no difference in NK cell activity compared to the control PBS treated mice. The NK cell activity in PBS and IFN pre-treated mice was also identical. In mice that had been treated with TNF plus Ab 301 there was less NK cell activity compared to the PBS control. NK activity was also noticeably lower for mice that had been pre-treated with the TNF plus Ab 301 and IFN γ therapy compared to the PBS control and the IFN γ alone treatment. The NK cell activity for the TNF plus Ab 301 treated mice was however generally similar to that of the TNF plus Ab 301 and IFN γ treated animals. These results suggest that the TNF/Ab 301/IFN-induced treatment is restricting the growth of vaccinia virus earlier than day 2 thereby preventing the stimulation of NK cells to normal levels of activation. This is supported by table 3.8 (see chapter 3).

TABLE 5.3 The Natural Killer (NK) cell activity in the spleens of CBA/H mice treated 24 hours before Vaccinia virus infection (10^7 pfu/mouse) with various cytokine therapies.

	Effector : Target ratio (% Specific lysis; YAC-1 targets).		
Treatment	**2 : 1**	**7 : 1**	**20 : 1**
Not Infected	1.9 ± 0.4	2.9 ± 0.12	6.6 ± 0.7
PBS	6.0 ± 0.5	15.6 ± 0.9	31.5 ± 1.6
6 µg TNF	7.2 ± 1.3	19.8 ± 1.2	30.9 ± 1.3
10^5 U mIFN γ	6.8 ± 0.2	16.4 ± 0.8	31.9 ± 0.5
6 µg TNF + Ab 301	3.6 ± 0.4	5.5 ± 0.6	25.0 ± 1.1
6 µg TNF + Ab 301 + 10^5 U mIFN γ	3.4 ± 0.2	8.0 ± 0.7	16.9 ± 1.2

Cytotoxic T Lymphocyte (CTL) activity to vaccinia virus after TNF plus Ab 301 therapy.

CBA/H mice that had been treated with TNF ± Ab 301 and challenged with vaccinia virus 24 hours later had their spleens removed at day 6 post infection to estimate CTL activity. Figure 5.2 a) shows that mice pre-treated with TNF alone exhibited CTL activity not significantly different to that found for the PBS pre-treated controls. TNF plus Ab 301 pre-treated mice showed lower CTL activity at all effector:target ratios in comparison to both the PBS and TNF alone pre-treated mice. Again this indicates that in TNF plus Ab 301 pre-treated mice the vaccinia virus growth

is controlled to such a degree that CTL activity is not fully stimulated. In the non-infected target cells none of the above described treatments induced non-specific CTL activity greater than 5 % (Fig. 5.2 b).

Profile of cell types in the peritoneal cavity of TNF ± Ab 301 treated and vaccinia infected mice.

Table 5.4 shows a differential profile of leucocytes recovered, at day 1 post-infection, from the peritoneal cavities of TNF pre-treated and vaccinia virus infected mice. The percentage of lymphocytes increased in the samples from PBS, TNF alone and TNF plus Ab 301 pre-treated, vaccinia infected mice compared to the samples from the non-treated/non-infected controls. There was no difference seen in the percentage of lymphocytes when the TNF alone pre-treated samples were compared to the TNF plus Ab 301 pre-treated lavage samples. The TNF plus Ab 301 treatment did not greatly differ in lymphocyte percentage compared to the PBS pre-treated controls. There was a noticeable difference in lymphocyte percentage however between the TNF alone group and the PBS group. Macrophage/monocyte percentages also did not show great variation between the vaccinia virus infected groups. Also no outstanding variation in the percentages of polymorphonuclear cells was observed between any of the groups whether infected with vaccinia virus or not.

At day 1 post-infection the number of peritoneal cells recovered from the virus-infected treatments were higher when compared to the non-infected controls. However, there were no significant variations in peritoneal cell number between PBS, TNF or TNF plus Ab 301 treated mice which had been vaccinia virus infected (data not shown).

TABLE 5.4 Differentiation of cells, on day 1 post-infection, from the peritoneal cavities of CBA/H female mice pre-treated with PBS, hTNF or hTNF plus Ab 301 and infected with 10^7 pfu vaccinia virus 24 hours later.

	Mean percentage ± SEM		
Treatment	**Macrophages/ monocytes**	**Lymphocytes**	**Polymorpho-nuclear cells**
Not treated /not infected	75.0 ± 4.7	24.0 ± 5.7	1.0
PBS	64.3 ± 2.7	34.0 ± 2.1	1.7 ± 0.9
6 µg hTNF	58.0 ± 1.7	40.7 ± 1.5	1.3 ± 0.3
6 µg hTNF + Ab 301	62.3 ± 1.8	36.7 ± 2.6	1.0

(n = 3) for each treatment.

The cells were stained with Diff-Quick. This stain does not give reliable staining of basophils and eosinophils.

The detection of non-specific antiviral activity in peritoneal lavage samples from TNF or TNF plus Ab 301 treated and vaccinia virus infected mice.

Table 5.5 summarises the antiviral effect of supernatants derived from peritoneal washings of TNF ± Ab 301 treated and vaccinia virus infected mice. HSV-1 was used as the indicator virus in this assay as VSV, which is routinely used elsewhere in bio-assays for IFN activity, is not available in Australia.

The results show that between the PBS pre-treated mice, the TNF alone and TNF plus Ab 301 treated mice there is no obvious increase in antiviral effect. The non-infected controls show only a very slight increase in HSV-1 titre in comparison to

samples from the vaccinia virus infected mouse groups.

TABLE 5.5 Absence of a non-specific antiviral state by U.V. irradiated peritoneal washings from CBA/H female mice that had been treated with hTNF or hTNF plus Ab 301 24 hours before infection with 10^7 pfu vaccinia virus. Reduction of HSV-1 titre was used to measure this activity.

Treatment	**Mean log $_{10}$ virus titre (pfu/ml) ± SEM**
Not treated/not infected	5.84 ± 0.05
PBS	5.72 ± 0.04
6 μg hTNF	5.75 ± 0.01
6 μg hTNF + Ab 301	5.75 ± 0.05

(n = 3) for each treatment.

Reactive Nitrogen Intermediate (RNI) levels in the peritoneal cavities of mice treated with TNF or TNF plus Ab 301 before vaccinia virus infection.

The levels of RNI in the peritoneal cavities of CBA/H mice treated with TNF alone or TNF in complex with Ab 301 are shown in table 5.6. The data shows that compared to controls there was no significant increase in RNI activity in samples from PBS, TNF alone or TNF plus Ab 301 treated mice that had been infected with vaccinia virus.

TABLE 5.6 Levels of reactive nitrogen intermediates (RNI) in the peritoneal washings of female CBA/H mice treated with hTNF ± Ab 301 24 hours before infection with 10^7 pfu vaccinia virus.

Treatment	Mean RNI (μM) ± SEM
Not treated/not infected	9.27 ± 0.92
PBS	8.24 ± 0.15
6 μg hTNF	9.72 ± 1.67
6 μg hTNF + Ab 301	9.56 ± 0.59

(n = 3) for each treatment.

SUMMARY.

Chapter 5 examined the host-immune response to vaccinia virus in mice pretreated with TNF plus Ab 301. The antiviral effect induced by TNF plus Ab 301 (with and without IFN γ) was not sensitive to sub-lethal gamma irradiation, indicating that a proliferative immune cell was not responsible for the observed attenuated vaccinia virus growth. This was further evidenced by NK cell and CTL studies which showed, when compared to controls, TNF plus Ab 301 treated animals had decreased spleen NK cell and CTL activities. This suggests, along with data in chapter 3, that a non-specific factor, effective earlier than day 2 post-infection, is responsible for the restriction of vaccinia virus growth. Enhanced IFN nor RNI activity could not be detected in the peritoneal cavities of TNF plus Ab 301 pretreated and vaccinia virus infected mice at day 1 post-infection.

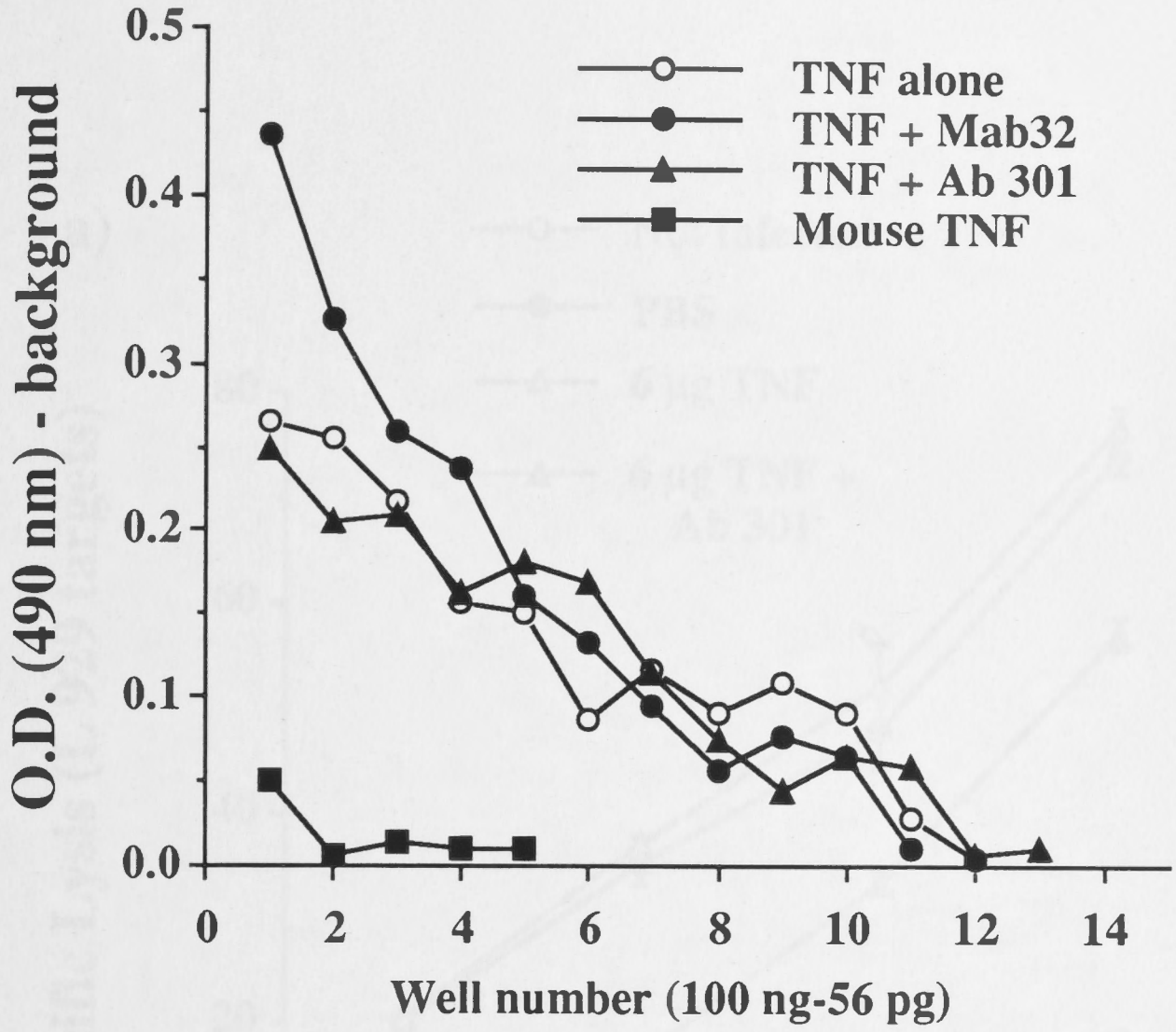


Figure 5.1 Comparison of the binding of hTNF alone to hTNF in complex with Mab 32 or Ab 301 in a sandwich ELISA assay.

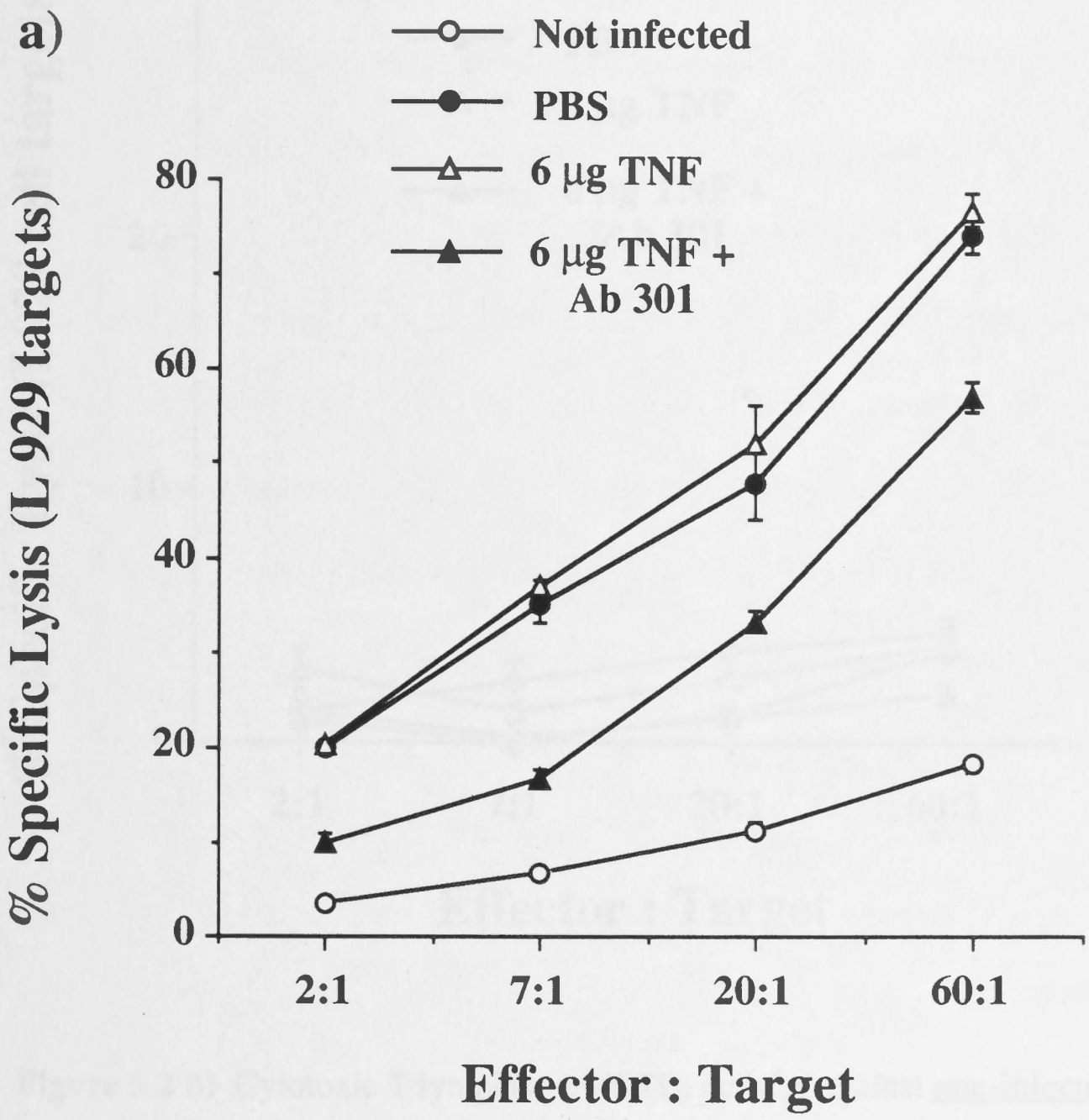


Figure 5.2 a) Cytotoxic T lymphocyte (CTL) activity against vaccinia virus infected (MOI = 20) L929 target cells in the spleens of mice that had been treated with TNF alone or TNF in complex with Ab 301 24 hours before vaccinia virus infection.

b)

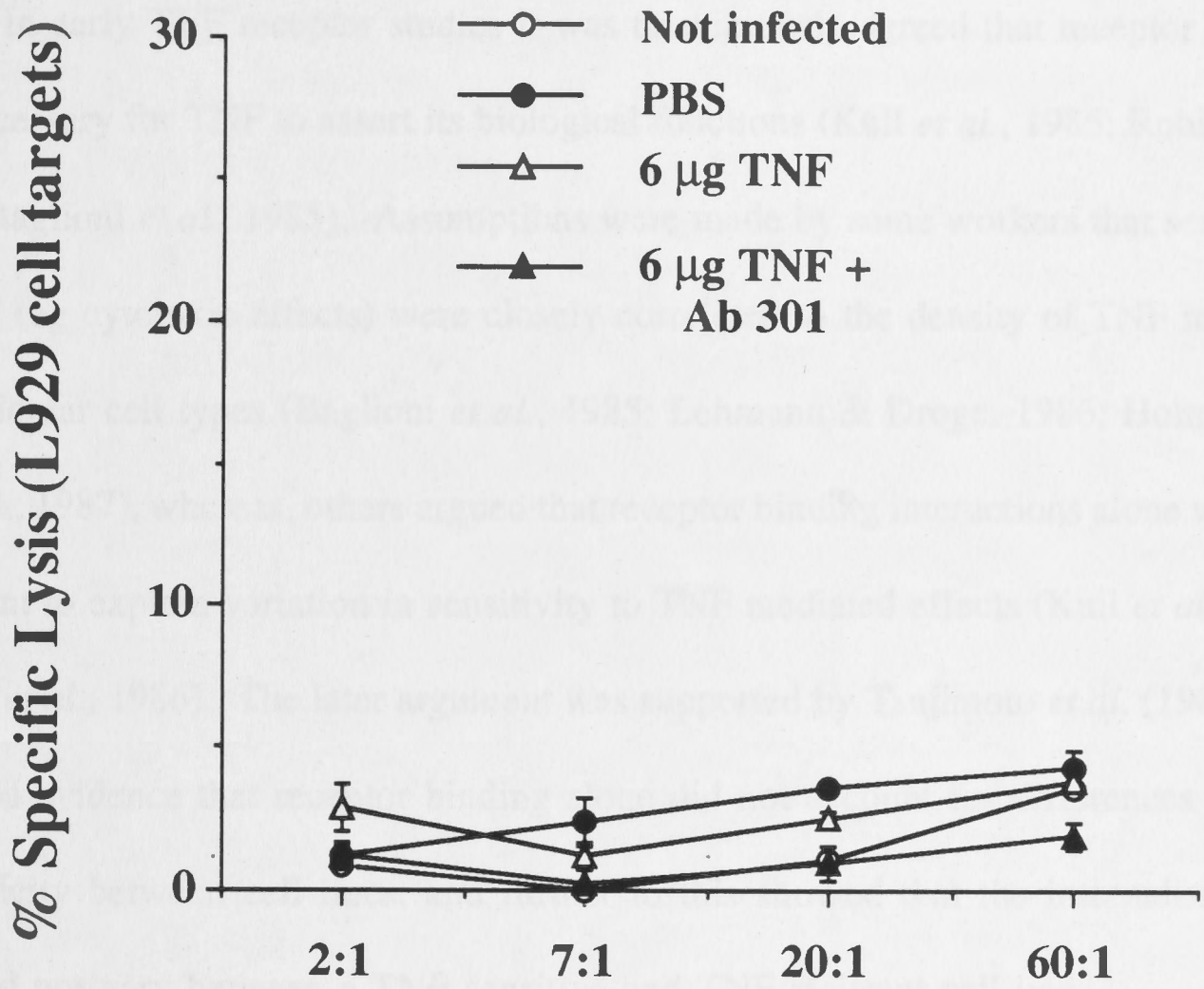


Figure 5.2 b) Cytotoxic T lymphocyte (CTL) activity against non-infected L929 target cells in the spleens of mice that had been treated with TNF alone or TNF in complex with Ab 301 24 hours before vaccinia virus infection.

Chapter 6.

The Effect of Enhancing Antibodies on TNF, TNF-Receptor Interactions.

INTRODUCTION.

In early TNF receptor studies it was unanimously agreed that receptor binding was necessary for TNF to assert its biological functions (Kull *et al.*, 1985; Rubin *et al.*, 1985; Baglioni *et al.*, 1985). Assumptions were made by some workers that sensitivity to TNF (eg cytotoxic effects) were closely correlated to the density of TNF receptors on particular cell types (Baglioni *et al.*, 1985; Lehmann & Droge, 1986; Holtmann & Wallach, 1987), whereas, others argued that receptor binding interactions alone were not sufficient to explain variation in sensitivity to TNF mediated effects (Kull *et al.*, 1985; Yoshie *et al.*, 1986). The later argument was supported by Tsujimoto *et al.* (1985) who provided evidence that receptor binding alone did not account for differences in TNF cytotoxicity between cell lines, and further to this showed that the internalisation of TNF did not vary between a TNF sensitive and TNF resistant cell line.

For all of the above evidence it was assumed that only one major class of TNF receptor existed. Many groups employed radiolabelled TNF cross-linking techniques to study TNF receptor properties and subsequently found varying sizes of cross-linked products according to cell type (Yoshie *et al.*, 1986; Tsujimoto *et al.*, 1986; Creasey *et al.*, 1987; Bakouche *et al.*, 1988). The discovery of a second major class of TNF receptor (Hohmann *et al.*, 1989) provided further insights into TNF:receptor relationships and may account for some of the differences seen in TNF activities. A review of the 2 known TNF receptors has been recently published (Tartaglia & Goeddel, 1992).

This chapter examines binding properties of TNF to its receptor(s) where TNF

is in complex with the specific enhancing antibodies described earlier. Two cell lines with different sensitivities to TNF antiviral effects were examined in this context. Also, the expression of receptors to TNF in cells that had been pretreated with TNF and subsequently virus infected will be discussed.

MATERIALS and METHODS.

Radio-receptor assay for L929 cells and HeLa cells.

Cells were harvested by trypsinisation, diluted to the concentration of 1 x 10^7 cells per ml in EMEM plus 1 % heat-inactivated FCS and kept on ice until required. Human TNF was iodinated (Iodine-125, [^{125}I]; Amersham, 100 mCi/ml) by a lactoperoxidase method which has been previously described (Aston *et al.*, 1985). The stock ^{125}I-TNF was diluted to approximately 50 000 CPM / 50 µl for the assay. To triplicate sets of tubes non-iodinated ("cold") TNF in 100 µl of EMEM containing 1 % FCS was added at the concentrations of 1, 10, 100, 500, 1000, 5000 and 10 000 ng per tube. To another set of triplicate tubes 100 µl of Mab 32 (5.0 mg/ml) or Ab 301 (5.0 mg/ml) serially diluted in EMEM (1/10 - 1/100 000) was added. To these tubes 50 µl of ^{125}I-TNF was added followed by 200 µl of L929 cells or HeLa cells. The tubes were vortexed and incubated for 3 hours at 37^{0}C (with periodic mixing). After the incubation period had elapsed 1 ml of EMEM-FCS was added, the tubes vortexed and then centrifuged (Beckman, TJ-6) for 5 minutes at 1600 g. The supernatants were discarded and as much residual media as possible drained from the cell pellets before counting the tubes in a Packard-Auto Gamma counter. Background levels of radiation were determined by control tubes which were treated exactly as described above except that no cells were added.

Scatchard Analysis of radio-receptor assay data.

Calculations to determine the amount of ^{125}I-TNF bound to the cells (B_{max}) and the binding affinity (K_d) of this radio-ligand were performed using the principles originally described by Scatchard (1949). For the ^{125}I-TNF alone treatment the above parameters were determined by examining the amount of radio-labelled ligand displaced at the various concentrations of non-iodinated (cold) TNF. The binding of ^{125}I-TNF in the presence of 10 000 ng cold TNF represented the non-specific binding. For the ^{125}I-TNF plus Mab 32 treatments the B_{max} and K_d were calculated on the basis of the extra quantity of ^{125}I-TNF binding to the cells at each Mab 32 dilution (based on the B_{max} value calculated for ^{125}I-TNF alone binding). For ease of calculation the data was linearised. The method for this as well as the methods for B_{max} and K_d determination were performed according to the procedures of Baxter and Funder (1979) and Munson and Enna (1984).

Internalisation of ^{125}I-TNF ± Mab 32.

Studies on the internalisation of hTNF ± Mab 32 were done according to the method described by Tsujimoto *et al.* (1985). The Mab 32 (5.0 mg/ml) was diluted 1/10 for this assay. Briefly, this method takes advantage of the property that at 4^{0}C ^{125}I-TNF will bind to its cellular receptors but will not become internalised. After an initial 4^{0}C binding period the cells were washed and then allowed to warm to 37^{0}C, whereafter, at time intervals of 1, 2 and 3 hours, samples were treated with a pH 3.0 glycine.HCl buffer to remove external receptor:^{125}I-TNF complexes. The CPM of the pellet, which had been solubulised in 0.1 % sodium dodecyl sulphate (SDS), represented the internalised ^{125}I-TNF.

Studies on TNF receptor expression post hTNF treatment and/or HSV-1 infection.

L929 cells (5 x 10^5 cells / well) in RPMI containing 5 % FCS were seeded into the wells of Linbro 24 well plates with TNF at the doses of 10, 100 and 400 ng in the presence or absence of Mab 32. TNF and Mab 32 were complexed at a ratio of 4 µg TNF to 1.0 mg Mab 32 for 1 hour at room temperature before this stock TNF:Mab 32 complex was diluted to the appropriate TNF doses and added to the wells of the L-24 plate. Some TNF treatments were also performed in the presence of 5000 U IFN γ. Twenty-four hours later the TNF ± IFN containing medium was removed and HSV-1 (MOI = 1.0) in 100 µl PBS or 100 µl PBS alone (mock infection) was absorbed onto the cell monolayers for 1 hour at 37°C. The HSV-1 containing PBS was then removed and fresh medium put into each well. Eighteen hours later the cells were scraped from the wells and placed into plastic counting tubes washed once and reconstituted with 300 µl of medium. Fifty microlitres of ^{125}I-TNF (50 000 CPM / 50 µl) was then added, the tubes mixed and incubated at 37°C for 3 hours with periodic agitation. After the incubation period had elapsed 1 ml of EMEM-FCS was added and the tubes centrifuged (Beckman, TJ-6) for 5 minutes at 1600 g. The supernatants were discarded and as much residual media as possible drained from the cell pellets before counting the tubes in a Packard-Auto Gamma counter. Background levels of radiation were determined by control tubes which were treated exactly as described above except that no cells were added.

* The experiments performed for this chapter were all done at least twice unless otherwise indicated. Please note that (n =) refers to the number of animals or samples in an experimental or control group.

RESULTS.

The binding of ^{125}I-TNF to L929 cells and HeLa cells is increased in the presence of Mab 32 and Ab 301.

Figure 6.1 a) shows the binding of ^{125}I-TNF either alone or in the presence of either Mab 32 or Ab 301 to L929 cells *in vitro*. Figure 6.2 b) describes the same effect of Mab 32 on TNF binding to HeLa cells. For both cell lines the presence of Mab 32 with ^{125}I-TNF resulted in a dramatic increase in the binding of this radio-labelled ligand to the cells. Ab 301 at higher concentrations (Fig. 6.1 a) enhanced the ^{125}I-TNF binding, although the increase of binding in the presence of Ab 301 was not as dramatic as that for Mab 32.

Scatchard analysis of ^{125}I-TNF binding with or without Mab 32.

The Scatchard plots for the binding of ^{125}I-TNF alone and ^{125}I-TNF plus Mab 32 to L929 cells are described in Figs. 6.2 a) and 6.2 b) respectively. The single line generated to describe the ^{125}I-TNF alone binding indicates that on L929 cells human TNF is binding to one class of high affinity receptor only. In the presence of Mab 32 the TNF binding is represented as a curve. A major class of high affinity receptor is represented as the steeper of the 2 lines which can be extrapolated to intersect the x-axis. The second line which does not intersect the x-axis usually indicates either the presence of a second low affinity TNF receptor or a high level of background radiation. The dynamics of ^{125}I-TNF binding in the Scatchard analysis for HeLa cells (Figs. 6.3 a and 6.3 b) are similar to that found in the L929 cells. Again TNF alone binding is represented by a single line that indicates in HeLa cells human TNF is binding to one class of high affinity receptor only. In the presence of Mab 32 the Scatchard analysis

for HeLa cells is again represented by a curve with the bottom line not intersecting the x-axis.

The B_{max} and K_d values for the above-described Scatchard analyses are summarised in Table 6.1. The presence of Mab 32 increases the binding of ^{125}I-TNF to L929 cells by a factor of approximately 2.2 times. This extra bound TNF did not appear to greatly compromise the affinity of TNF for its receptor as indicated by similar K_d values. For HeLa cells the increase in TNF binding in the presence of Mab 32 is approximately 2.3 times greater than that of TNF alone. However, the enhanced binding of TNF with Mab 32 was associated with a change in the affinity of TNF for its receptor on HeLa cells as the K_d value for TNF plus Mab 32 is approximately three times higher than that of TNF alone.

TABLE 6.1 Scatchard analysis summary. Binding of ^{125}I-hTNF either alone or in the presence of Mab 32 to the surface of L929 cells and HeLa cells.

	L929 cells		HeLa cells	
Treatment	**B_{max}**	**K_d**	**B_{max}**	**K_d**
^{125}I-TNF alone	19.60 pM	2.59 pM	13.70 pM	1.14 pM
^{125}I-TNF + Mab 32	43.10 pM	3.07 pM	31.40 pM	3.70 pM

Internalisation of ^{125}I-TNF in the presence of Mab 32.

Figures 6.4 a) and 6.4 b) describe the comparison of ^{125}I-TNF internalisation with or without Mab 32 in L929 cells and HeLa cells respectively. For both cell lines the presence of Mab 32 greatly enhances the internalisation of ^{125}I-TNF. This occurs within the first hour of incubation for both L929 cells and HeLa cells. Over the 3 hour incubation period the levels of internalised ^{125}I-TNF for both TNF alone and TNF plus Mab 32 treatments remain relatively constant in both cell lines. The amount of ^{125}I-TNF internalised in the presence of Mab 32 was marginally higher in L929 cells, particularly at the 1 and 3 hour time points, when compared to the same treatments in HeLa cells. In the presence of a control monoclonal antibody (Mab 42) internalisation of ^{125}I-TNF into L929 cells was similar to that observed for the ^{125}I-TNF alone treatment (data not shown).

Post TNF (± IFN γ) treatment and HSV-1 infection expression of TNF receptors in L929 cells.

Pretreatment of L929 cells with TNF that were subsequently mock-infected led to a dose dependent increase of TNF receptors (TNF-R). At no dose of TNF used was there a significant enhancement of TNF-R expression by Mab 32 (Fig. 6.5 a). Fig. 6.5 b) shows that in cells that had been pretreated with 10 ng TNF (± Mab 32) and infected 24 hours later with HSV-1 (MOI = 1.0) there was a large increase of TNF-R expression compared to similarly treated cells that had been mock-infected (Fig. 6.5 a). Marginal increases for the 100 and 200 ng TNF (± Mab 32) treatments were also observed with HSV-1 infection. However, the infection of non-TNF treated L929 cells with HSV-1 also resulted in a dramatic increase in TNF-R expression very similar to that found for the TNF pretreated cells, suggesting that any increases of TNF-R expression seen

compared to the mock-infected cells are directly due to the HSV-1 infection.

Inclusion of IFN γ alone in the cultures for the first 24 hours of the assay significantly increased TNF-R expression in mock-infected cells compared to controls (Fig. 6.6 a). The treatments of TNF (10-200 ng) alone plus IFN γ did not significantly vary the TNF-R expression compared to the IFN γ alone treatment. When the cells had been pretreated, with 100 or 200 ng TNF in complex with Mab 32 plus IFN, greater levels of TNF-R expression were observed compared to IFN treatment alone. The TNF-R expression was significantly enhanced by Mab 32 at the 200 ng TNF dose.

In cells that had been pretreated with TNF ± Mab 32 and IFN and then infected with HSV-1 24 hours later (Fig. 6.6 b) the profile of TNF-R expression was different to that found for mock-infected cells. In cells treated with IFN alone before infection there was significantly less TNF-R expression in comparison to the cells that had only been infected with HSV-1. The level of TNF-R expression for these IFN-treated, HSV-1 infected cells was also lower than that found for cells that had been IFN treated and mock-infected. For all of the TNF alone plus IFN treatments there were small increases in TNF-R expression compared to that observed for cells that had only been infected with HSV-1. There were no significant differences observed in TNF-R expression when the three TNF alone (plus IFN) doses were compared. There was a notable, but not significant decrease in TNF-R expression for the 10 ng TNF plus Mab 32 (plus IFN) treatment compared to the 10 ng TNF alone (plus IFN) treatment, whereas at the 100 and 200 ng TNF doses (plus IFN) significant enhancement of TNF-R expression was observed if Mab 32 was present.

SUMMARY.

The presence of Mab 32 with TNF enhances both the binding of TNF to the surface of L929 cells and HeLa cells *in vitro*, as well as enhancing the internalisation of this ligand into both of these cell lines. The enhancement of these properties are very similar for both cell lines with the only possible difference in binding characteristics being that the presence of Mab 32 slightly compromises the binding affinity of TNF to the HeLa cell TNF receptor (TNF-R). These overall similarities in TNF binding and internalisation properties are particularly interesting in relation to the sensitivities of these 2 cell lines to TNF antiviral activity (ie, L929 cells are sensitive; HeLa cells are resistant).

The expression of TNF-R in TNF and/or IFN γ exposed cells, both with and without HSV-1 infection, was examined. TNF alone could induce a dose dependent increase in TNF-R which was not significantly increased by Mab 32. IFN γ alone also induced TNF-R expression. HSV-1 infection of L929 cells resulted in a significant upregulation of TNF-R expression which was partially reversed if the cells had been pre-exposed to IFN γ. The pre-exposure of L929 cells to TNF plus IFN γ before infection did not show any dramatic effects except for the significant enhancement of TNF-R expression if exogenous TNF (100 and 200 ng doses) was present with Mab 32.

a)

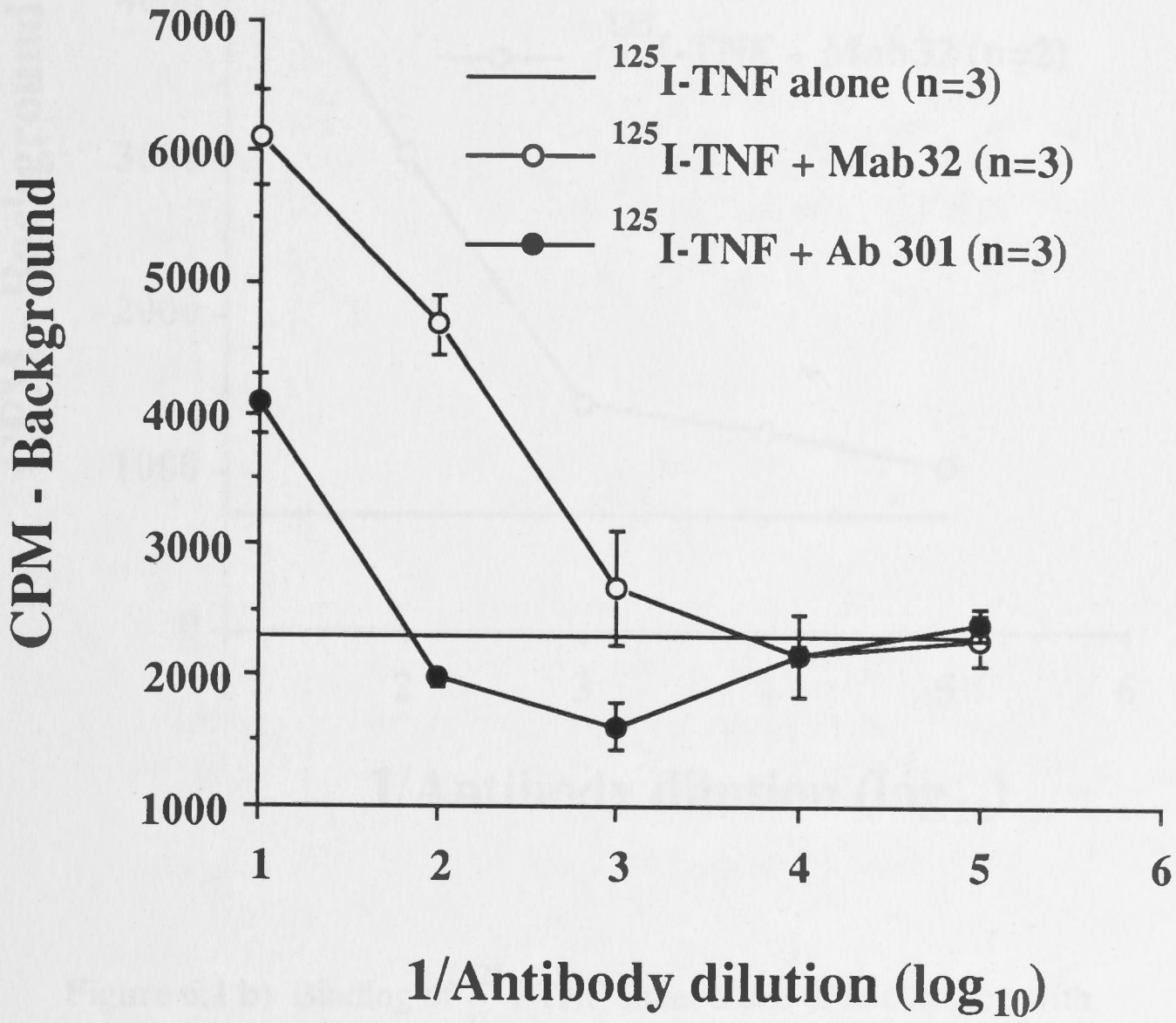


Figure 6.1 a) Binding of ^{125}I-TNF either alone or in complex with Mab 32 or Ab 301 to L929 cells.

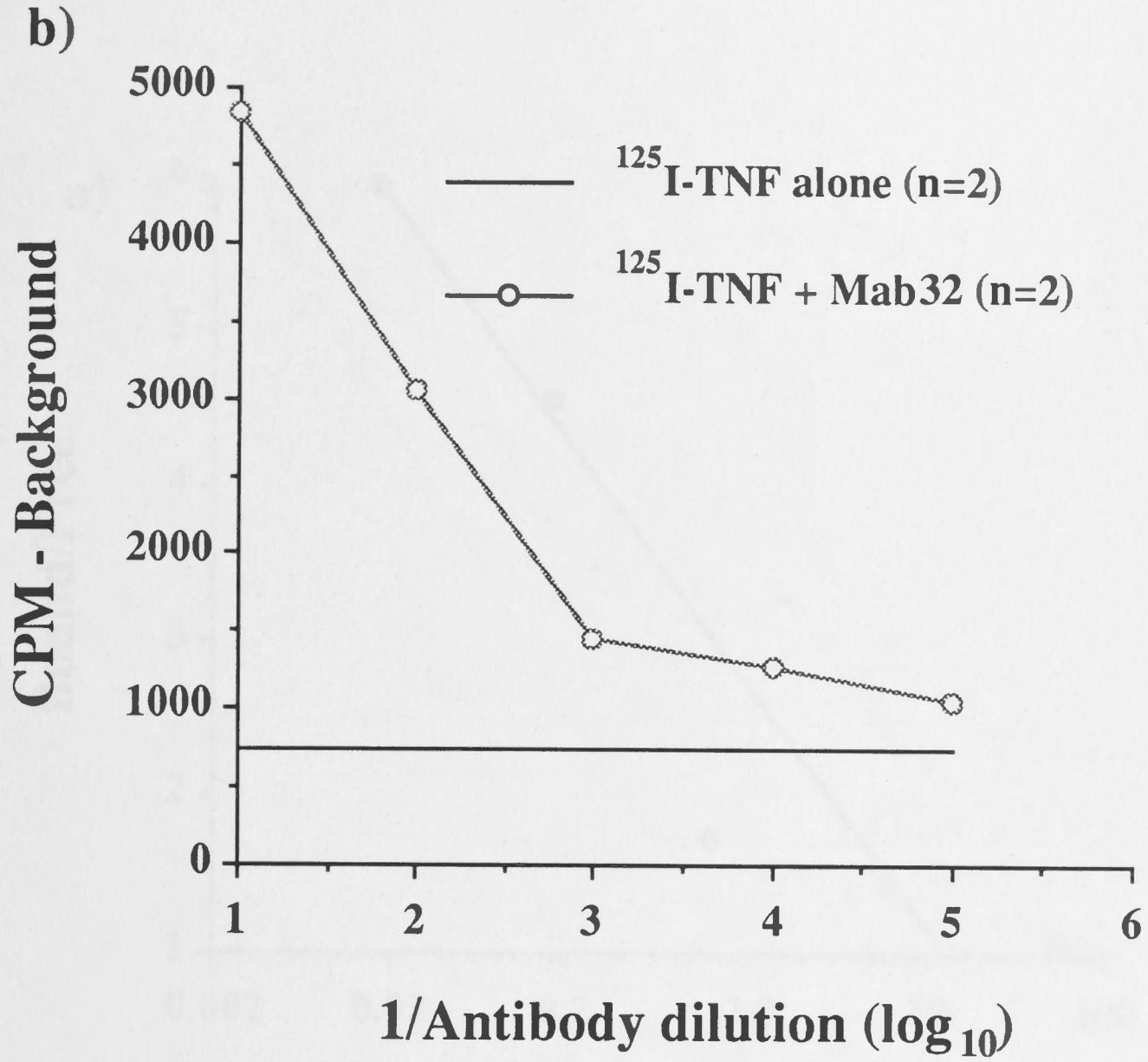


Figure 6.1 b) Binding of ^{125}I-TNF either alone or in complex with Mab 32 to HeLa cells.

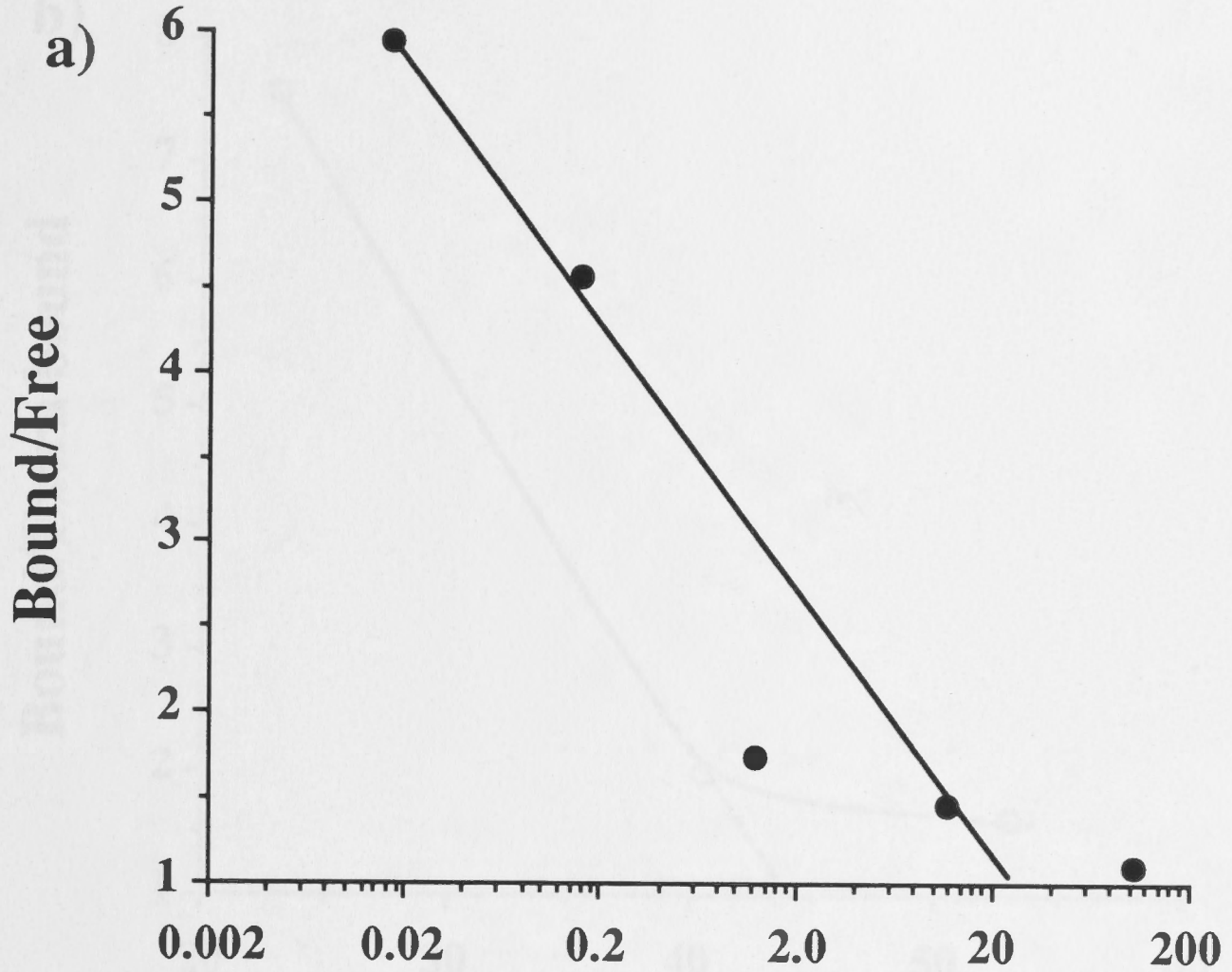


Figure 6.2 a) Scatchard analysis of ^{125}I-TNF alone binding to L929 cells.

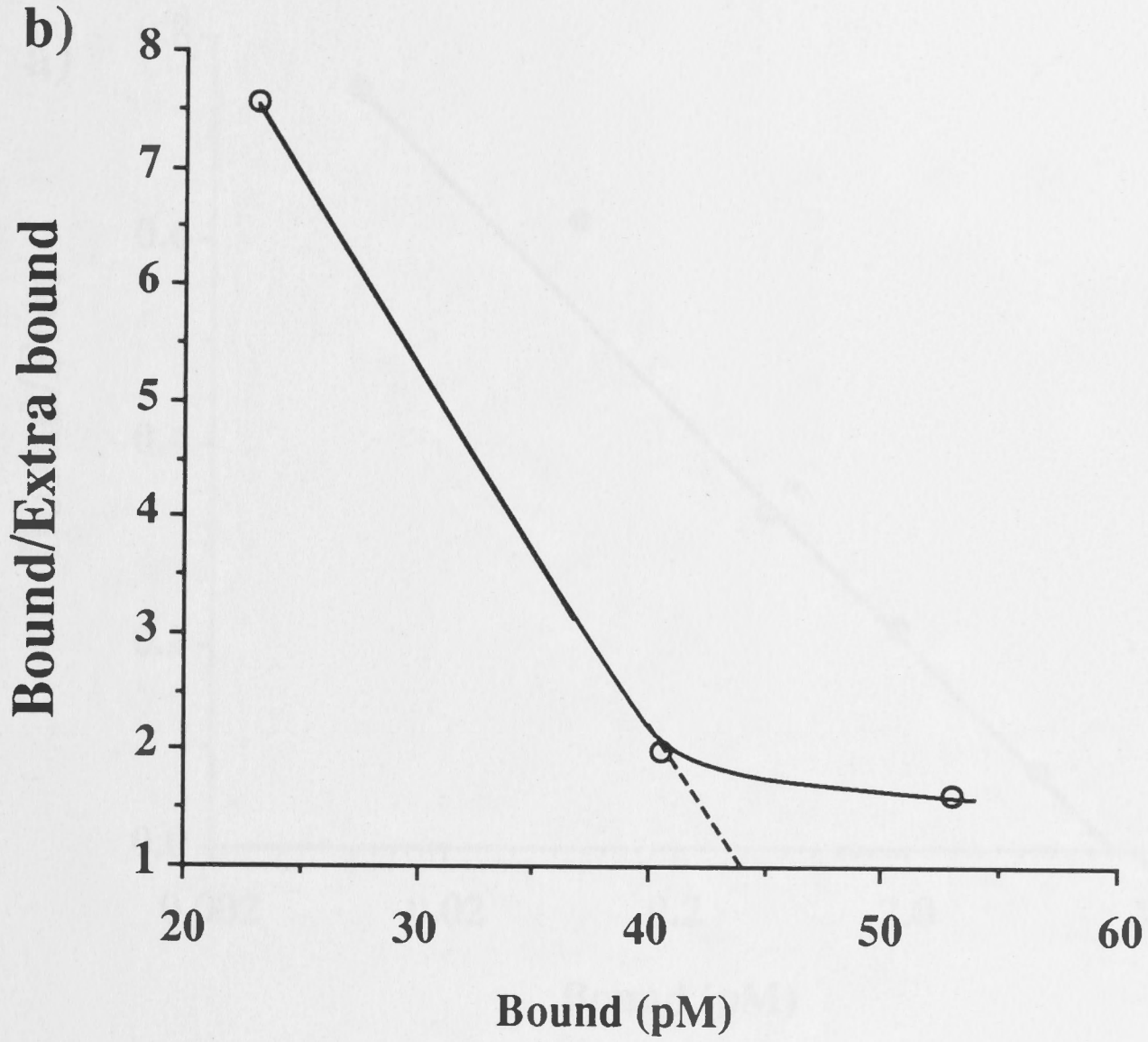


Figure 6.2 b) Scatchard analysis of ^{125}I-TNF plus Mab 32 binding to L929 cells.

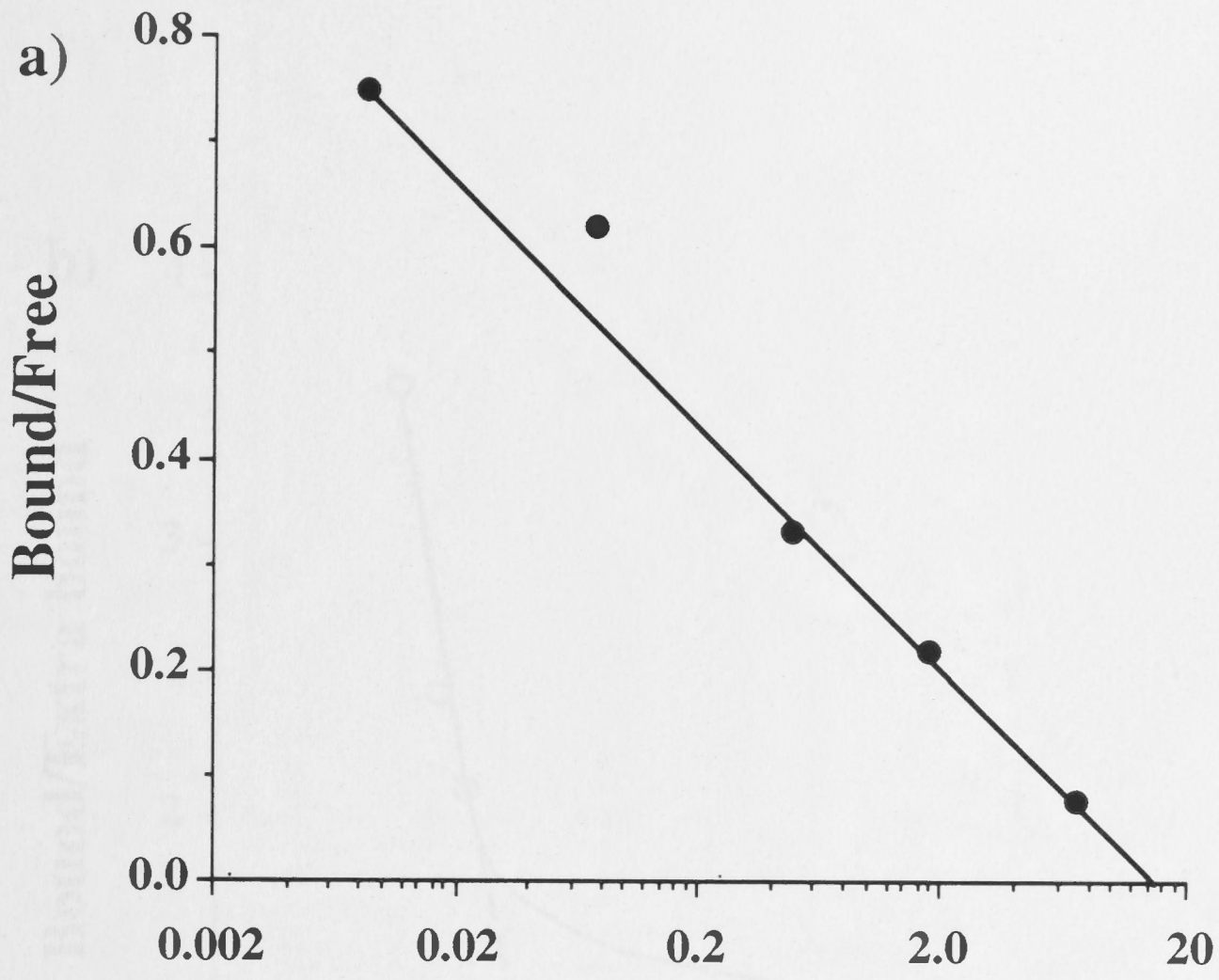


Figure 6.3 a) Scatchard analysis of ^{125}I-TNF alone binding to HeLa cells.

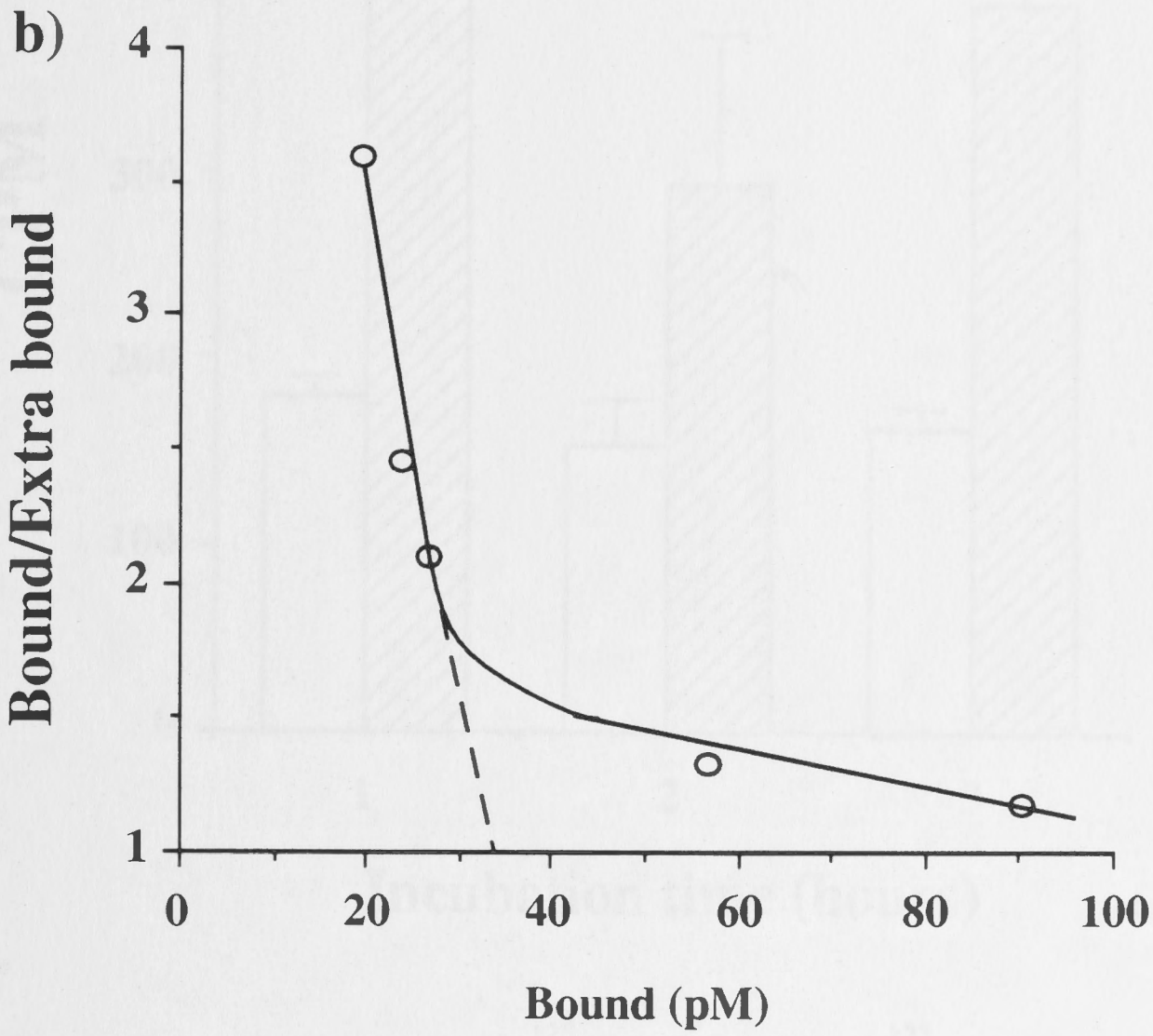


Figure 6.3 b) Scatchard analysis of ^{125}I-TNF plus Mab 32 binding to HeLa cells.

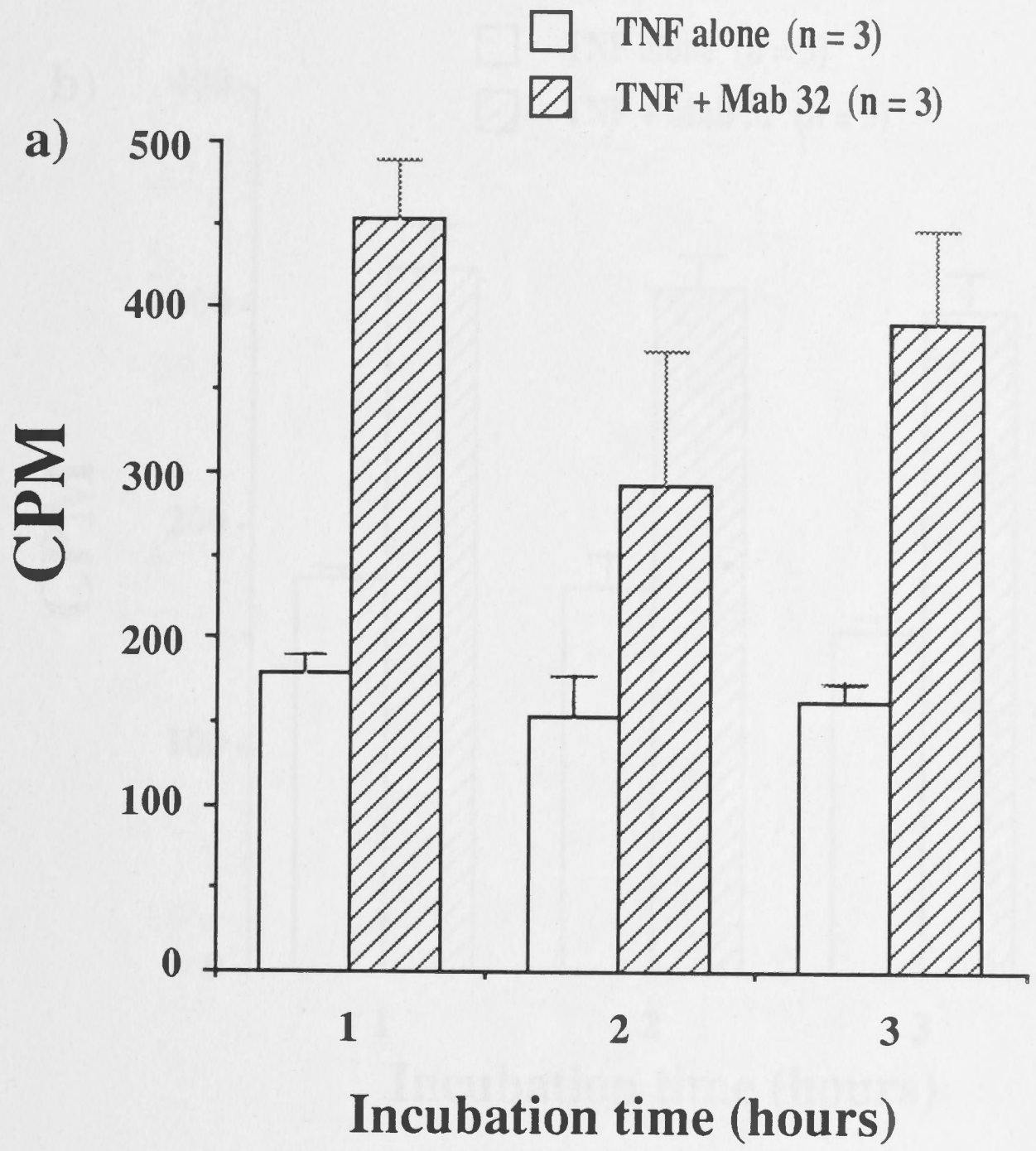


Figure 6.4 a) Internalisation of ^{125}I-TNF alone versus ^{125}I-TNF plus Mab 32 in L929 cells.

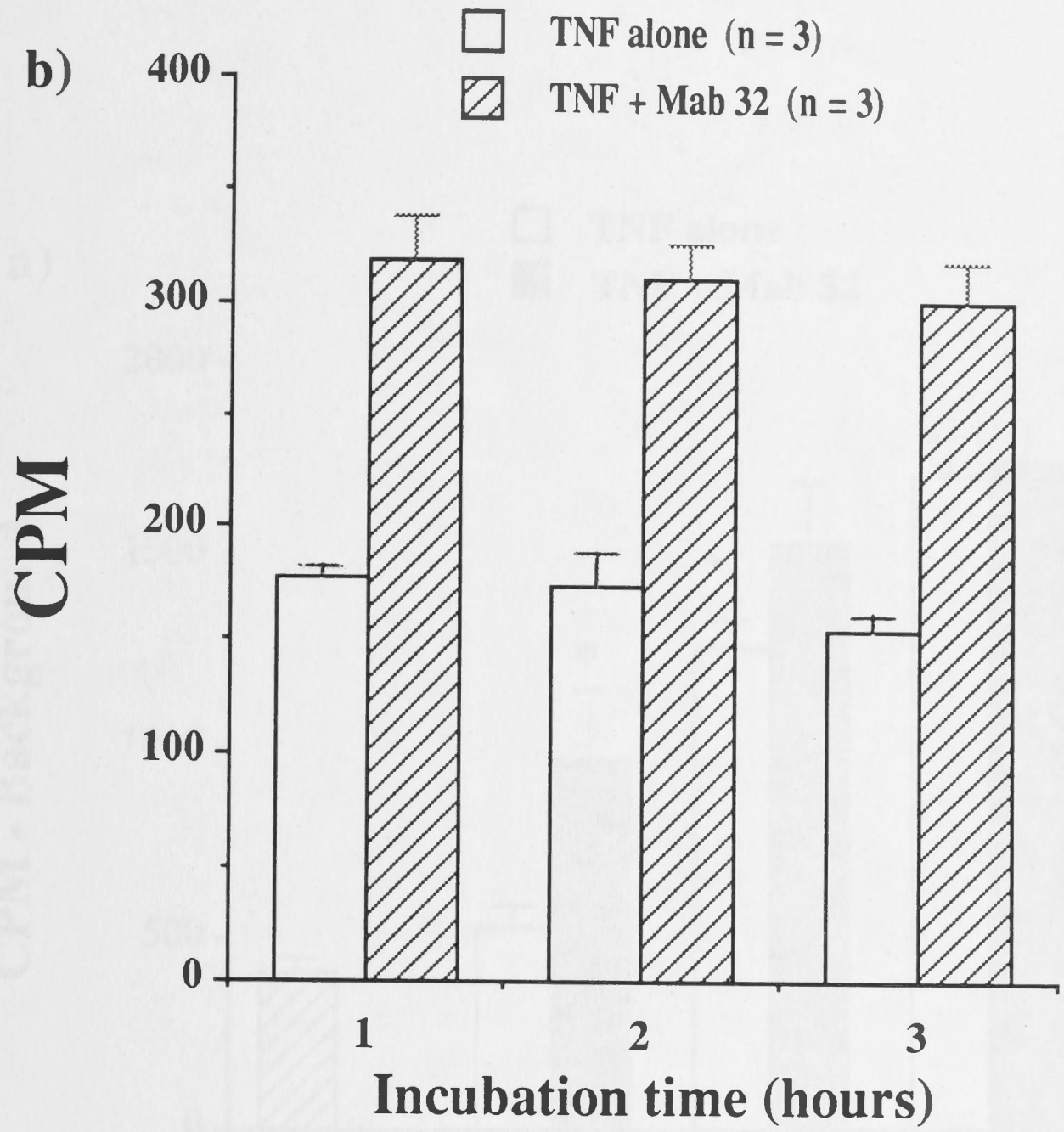


Figure 6.4 b) Internalisation of ^{125}I-TNF alone versus ^{125}I-TNF plus Mab 32 in HeLa cells.

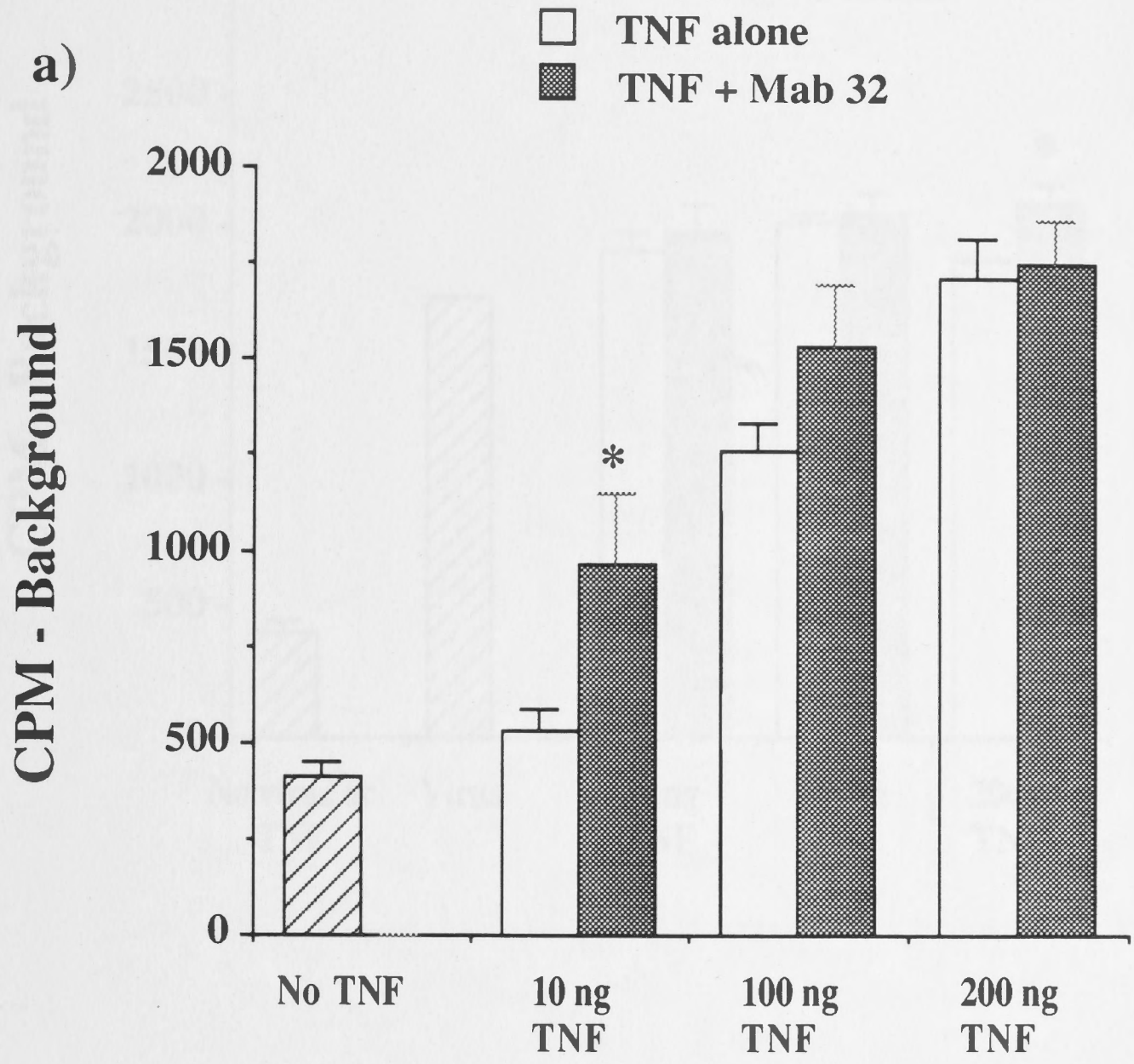


Figure 6.5 a) Expression of TNF receptors in L929 cells that had been TNF ± Mab 32 pretreated and mock-infected. (n = 3) for each treatment.

* p = 0.089 versus the TNF alone treatment at the same dose.

b)

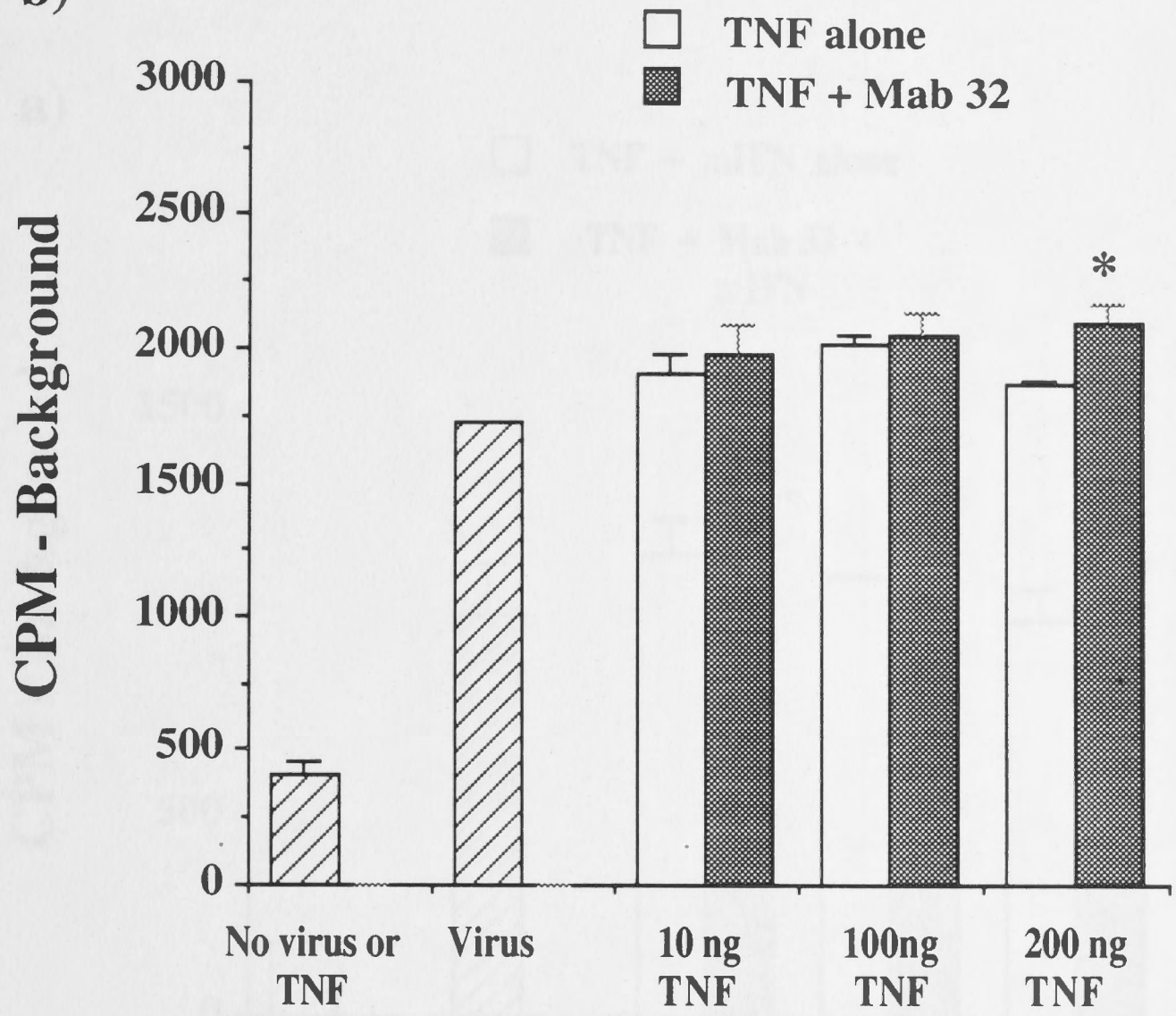


Figure 6.5 b) Expression of TNF receptors in L929 cells that had been TNF ± Mab 32 pretreated and HSV-1 (MOI = 1.0) infected. (n = 3) for each treatment.

* p = 0.05 versus the TNF alone treatment at the same dose.

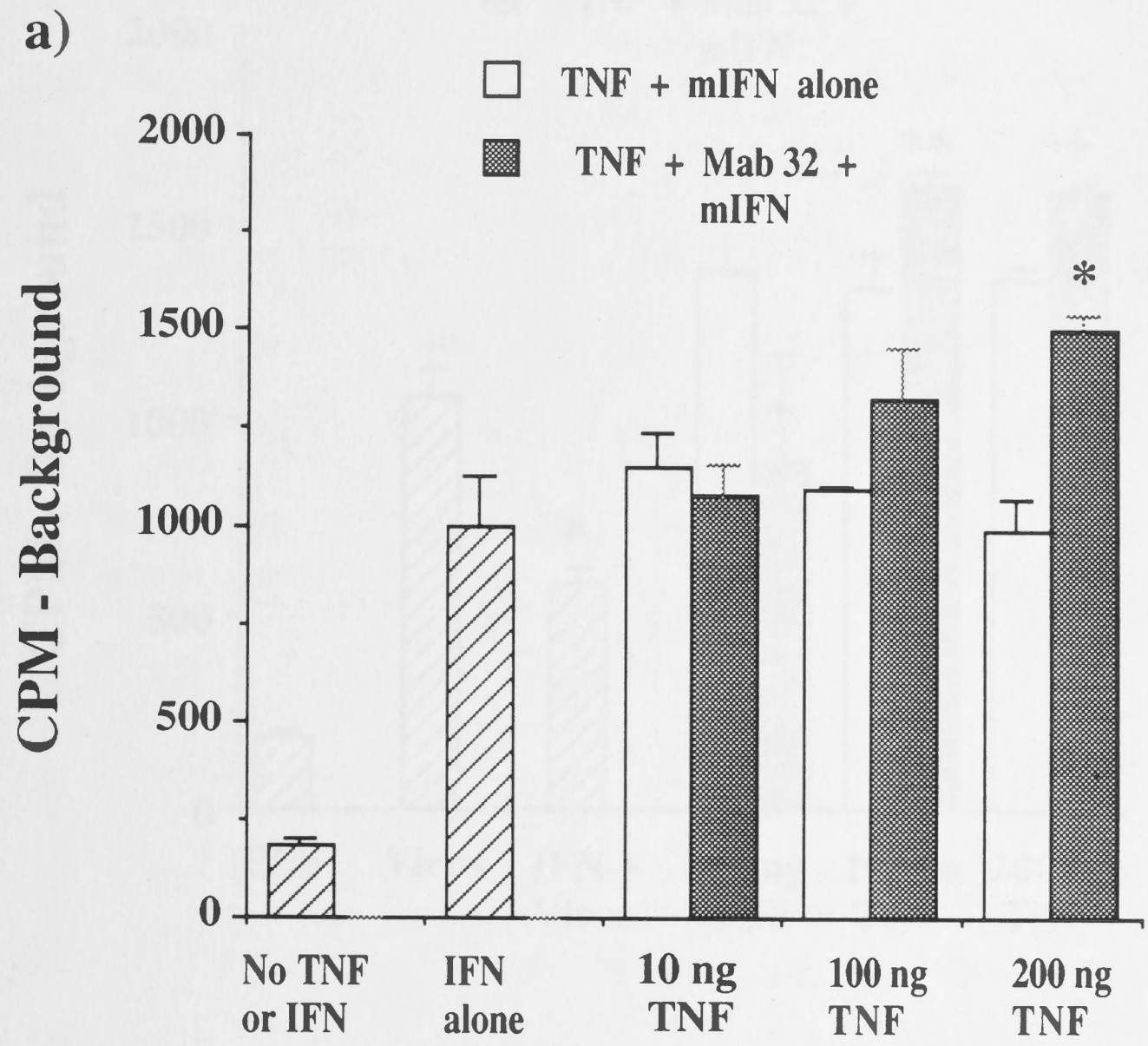


Figure 6.6 a) Expression of TNF receptors in L929 cells that had been pretreated with TNF ± Mab 32 as well as 5000 U mIFN γ and mock-infected. (n = 3) for each treatment.

* p = 0.005 versus TNF alone treatment at the same dose.

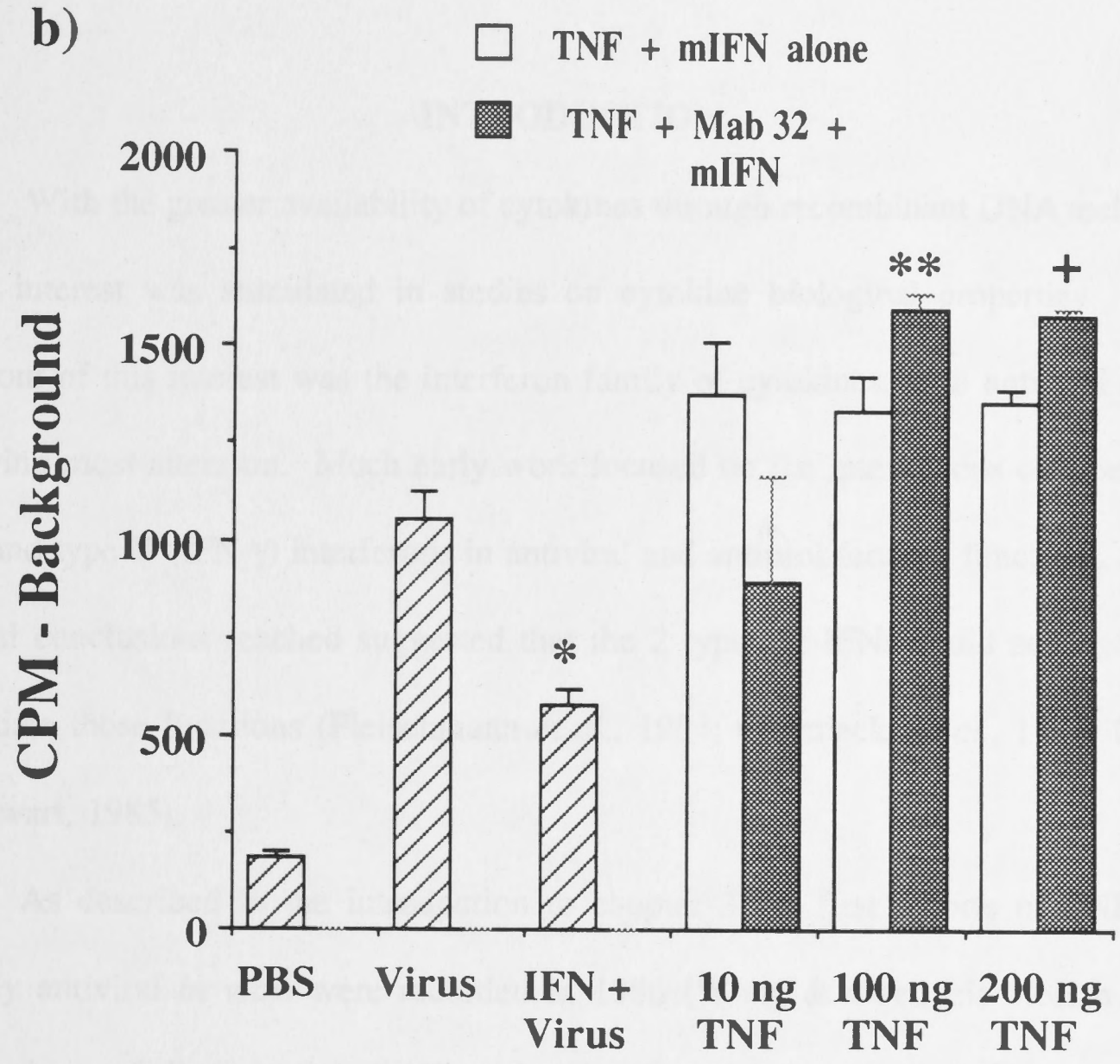


Figure 6.6 b) Expression of TNF receptors in L929 cells that had been pretreated with TNF ± Mab 32 as well as 5000 U mIFN γ and HSV-1 (MOI = 1.0) infected. (n = 3) for each treatment.

* p = 0.004 versus HSV-1 treatment alone.

** p = 0.052 versus the TNF treatment alone at the same dose.

\+ p = 0.005 versus the TNF treatment alone at the same dose.

Chapter 7.

The Enhancement by Antibodies of the Direct Antiviral Efficacy of TNF *in vitro*.

INTRODUCTION.

With the greater availability of cytokines through recombinant DNA technology, much interest was stimulated in studies on cytokine biological properties. At the forefront of this interest was the interferon family of cytokines, with antiviral activity receiving most attention. Much early work focused on the interactions of type I (IFN α/β) and type II (IFN γ) interferons in antiviral and antiproliferative functions, and the general conclusions reached suggested that the 2 types of IFNs could act together to potentiate those functions (Fleischmann *et al.*, 1984; Czarniecki *et al.*, 1984; Oleszak & Stewart, 1985).

As described in the introduction to chapter 3 the first reports of TNF being directly antiviral *in vitro* were recorded in 1986 (Wong & Goeddel; Mestan *et al.*). Mechanisms of direct antiviral effects by interferons had, at this stage, been largely determined. With the discovery of another directly antiviral cytokine, which synergised with type II IFN, much interest resulted in the mechanisms by which TNF exerted this effect. The prime interest of many investigators was the induction of TNF mediated antiviral effects via IFN or IFN-induced antiviral pathways, for example, 2-5A synthetase. In Wong and Goeddel's original paper on TNFs direct antiviral effects they maintained that IFN-induction was not important in this effect, and while 2-5A synthetase was induced its presence did not correlate with observed TNF antiviral effects. Over ensuing years studies on the role of IFNs and IFN-induced compounds nevertheless continued with much interest. This area of past research is fully discussed

in chapter 1 of this thesis.

The primary interest of this chapter is to examine whether the documented direct *in vitro* antiviral properties of TNF can be enhanced by the antibodies which, as described earlier, potentiated TNFs *in vivo* antiviral effects. Further to this, evidence of an alternative mechanism of TNF-mediated virus growth restriction *in vitro* is presented in the context of both vaccinia virus and HSV-1 infection.

MATERIALS and METHODS.

In vitro antiviral assays.

L929 cells in RPMI plus 5 % FCS or HeLa cells in EMEM plus 5 % FCS were seeded into the wells (5 x 10^5 cells/ml/well) of Linbro 24 well (L-24) plates with TNF at the indicated doses in the presence or absence of Mab 32. TNF and Mab 32 was complexed at a ratio of 4 µg TNF to 1.0 mg Mab 32 for 1 hour at room temperature before this stock TNF:Mab 32 complex was diluted to the appropriate TNF doses and added to the wells of the L-24 plate. A control containing Mab 32 alone was also done. Twenty-four hours later the TNF containing medium was removed and HSV-1 (MOIs = 0.1 - 2.0) in 100 µl PBS was absorbed onto the cell monolayers for 1 hour at 37^{0}C. The HSV-1 was subsequently removed and fresh medium put into each well. The plates were then incubated for 48 hours at 37^{0}C in a humidified atmosphere containing 5 % CO_2 after which the plates were frozen and thawed twice. These cell lysates were serially diluted by a factor of 10 fold in PBS and the titre of virus determined via a plaque assay using Vero cells. For vaccinia virus the method was essentially the same except for the following differences: 1) the virus was sonicated before absorption onto

the L929 cells, 2) the cell lysates were incubated in 0.1 % trypsin for 30 minutes at 37^{0}C before titration and 3) the serial dilutions were done in HEPES-buffered saline containing 0.5 % gelatin.

(N.B. - A more comprehensive description of plaque assays for vaccinia virus and HSV-1 are to be found in chapters 3 and 4 respectively).

MTT cell cytotoxicity assays.

Twenty thousand L929 cells in RPMI plus 5 % FCS were seeded into the wells of a Linbro flat-bottomed 96 well plate with 0.4, 4.0, 8.0 or 16 ng of TNF ± Mab 32. The same TNF:Mab 32 ratio was used as described above. These plates were incubated for 24 hours at 37^{0}C. After incubation the TNF containing media were removed and the confluent L929 cells were either mock-infected (50 µl PBS alone) or infected with HSV-1 (MOI = 0.1) in 50 µl of PBS for 1 hour at 37^{0}C. The virus-containing media were removed, fresh media added and the plates incubated for 48 hours at 37^{0}C. At the completion of this incubation 10 µl of 5 mg/ml MTT (in PBS) was added and the plates incubated for 6 hours at 37^{0}C in a humidified atmosphere containing 5 % CO_2. After this the wells were treated with 100 µl of 10 % SDS in 0.01 M HCL and incubated overnight at 37^{0}C. The absorbances were then read at 600 nm (reference 630 nm) on a Dyna-Tech plate reader. It should be noted that unlike the standard MTT assay actinomycin D was not added.

Measurement of murine TNF.

Murine TNF was assayed via a specific sandwich ELISA technique. The capture antibody (TN3-19.12), a hamster anti-mouse TNF-α/β monoclonal antibody was coated onto the wells of a 96 well Nunclon maxisorp plate in a carbonate coating buffer, pH

9.6. The plates were left at 4^{0}C overnight. The plates were then blocked with PBS plus 3 % BSA and 0.1 % tween 20 for 2 hours at room temperature. The standards and samples were then added to the coated and blocked plates and incubated overnight at 4^{0}C. After the overnight incubation a polyclonal rabbit anti-mouse TNF probe antibody (raised in our laboratory by Sue Fordham) which had been diluted 1/500 was added and the plates incubated for 2 hours at room temperature. This was followed by the addition of an alkaline phosphatase conjugated sheep anti-rabbit Ig (Silenus, Hawthorn, Australia) which had been diluted to 1/1000. The plates were incubated for 60-90 minutes at room temperature. After this incubation had finished the alkaline phosphatase substrate was added (alk. phos. tablets [Sigma, St. Louis] dissolved in pH 9.8 diethanolamine/$MgCl_2$ buffer) and the plates then incubated at room temperature for 30-60 minutes. The absorbances were read on a Dyna-Tech automatic plate reader at 410 nm (reference = 630 nm). The plates were washed 6 times between each of the above steps with PBS plus 0.1 % tween 20. The dilution of samples, standards and antibodies were all done in PBS plus 1 % BSA and 0.1 % tween.

Measurement of interleukin-1 activity.

The L929 supernatants from infected wells were first U.V. irradiated* (5 minutes at 10 centimetres). These irradiated supernatants were then tested for IL 1 bioactivity in a D10.G4.1 cell proliferation assay that has been described previously (Kaye *et al.*, 1984; Ruby *et al.*, 1991).

Examination of L929 cellular DNA.

L929 cells were prepared exactly as described in the *in vitro* antiviral assay procedure; the TNF dose used was 400 ng. The cells were mock-infected or infected with HSV-1 at a MOI equal to 0.1. Four and forty eight hours[+] post infection the cells were scraped out of the wells, suspended in 1.0 ml PBS plus pronase buffer and incubated overnight with 50 µl 10 mg/ml pronase at 37^{0}C. Three million cells were harvested for each treatment. After the pronase treatment the DNA was extracted with 2 x phenol/chloroform treatments and then precipitated with a 2.5 x volume of cold absolute ethanol after which the tubes were stored at -70^{0}C overnight. The precipitated DNA was collected by centrifugation in an eppendorf microfuge for 30 minutes at 14 000 RPM. The DNA pellets were washed twice with 70 % ethanol and were then dried under vacuum. The dried pellets were rehydrated with 120 µl of 10 mM EDTA and kept at 4^{0}C overnight. These tubes were then treated with 4 µl of 20 mg/ml RNAase A and incubated for 4 hours at 37^{0}C. Sixteen microlitres of each sample was subsequently loaded into the wells of a 1.5 % agarose gel and the DNA electrophoretically separated at 50 volts for 2.5 hours in TAE buffer containing 0.05 mg/100 mls ethidium bromide.

+ Four hours is the time point at which apoptotic changes normally occur and 48 hours post-infection corresponded to peak CPE.

Detection of cytotoxicity in L929 cell culture supernatants.

L929 cells were seeded into L-24 plates as described above with 400 ng TNF ± Mab 32. The TNF-treated L929 cell monolayers were either mock-infected or infected with HSV-1 (MOI = 0.1) 24 hours later and incubated for 48 hours at 37^{0}C. After this time had elapsed the culture supernatants were removed from the L929 cells and U.V. irradiated* (5 minutes at 10 centimetres). Another set of 2 x 10^6 L929 cells

were incubated with ^{51}Cr (50 μCi / 2 x 10^6 cells) in a volume of 200 μl of RPMI plus 5 % FCS for 1.5 hours at 37°C. These cells served as targets for any cytotoxic factors present. One hundred microlitres of the U.V. irradiated L929 cell supernatants were added to the wells of a 96 well round bottomed Linbro microtitre plate. To these wells were then added 100 μl of RPMI plus 5 % FCS containing 2.0 x 10^4 thrice washed ^{51}Cr-labelled L929 cells. Plates were then incubated for 24 and 48 hours at 37°C in a humidified atmosphere containing 5 % CO_2. The supernatants were collected at the 24 and 48 hour time points, counted on a Packard Auto-Gamma counter and the specific lysis estimated exactly as described in chapter 5. Non-specific lysis at both 24 and 48 hours was approximately 25 %.

* Samples from irradiated supernatants were plated onto confluent Vero cells and found not to contain any active HSV-1.

Treatment of L929 cultures with butylated hydroxyanisole (BHA).

BHA (Sigma, St. Louis) was dissolved to the required concentration in 70 % ethanol. From this stock a concentration equivalent to 100 μmoles/ml of RPMI plus FCS was added. It has been suggested (Schulze-Osthoff *et al.*, 1992) that the concentration of the organic solvent in cell culture should not exceed 0.4 %. The concentration of the organic solvent in this case was less than 0.1 %.

L929 cells were treated with TNF and infected with HSV-1 (MOI = 0.1) in L-24 plates as described above. However in these experiments BHA (100 μmoles/ml/well) was added at 2 time points, ie, before HSV-1 infection (therefore the BHA was present at the same time as TNF) and immediately after the absorption of the virus onto the cell monolayers. These cultures were left for 48-72 hours at 37°C in a humidified

atmosphere containing 5 % CO_2. During this time the cultures were observed and photographed (Ilford Pan-F, ASA 50) when appropriate control levels of CPE had developed. Immediately after the photographs had been taken the cells were frozen and thawed twice and titrated on Vero cells as previously described.

The response of vaccinia virus to BHA treatment was also tested as described above for HSV-1. BHA was only included in these cultures after the vaccinia virus infection of the cell monolayers.

Treatment of L929 cultures with N^G Ethyl-L-Arginine acetate (NELA).

L929 cells were TNF-treated and infected with HSV-1 as described above. Immediately after infection relevant cultures were supplemented with RPMI plus 5 % FCS containing 50 µmoles/ml of NELA. The stock NELA was dissolved in RPMI containing 5 % FCS. The NELA was produced in our lab by a previously described method (Cho *et al.*, 1984).

Treatment of L929 cultures with cyclooxygenase/lipoxygenase inhibitors.

L929 cells were seeded into L-24 plates (without TNF) as previously described. This time however the cultures contained between 10-100 µmoles of one of the following cyclooxygenase/lipoxygenase inhibitors; ibuprofen (Sigma, St. Louis), BWA 137C (Huang *et al.*, 1989) or BW 755C (Challend *et al.*, 1981). All of these inhibitors were dissolved in propane diol (final concentration of propane diol in culture was approximately 0.1 %). Twenty-four hours later the L929 cells were infected with HSV-1 (MOI = 0.1) and fresh media containing the same quantities of the above described compounds placed into the corresponding wells. These culture were incubated at 37^{0}C for 48 hours, frozen and thawed twice and titrated on Vero cells as previously

described.

Detection of superoxide dismutase (SOD) in TNF-treated and virus-infected L929 cultures.

L929 cells were treated with 400 ng TNF alone or with Mab 32 and 24 hours later were either mock-infected (PBS alone) or infected with HSV-1 or vaccinia virus (MOI = 0.1) in L-24 plates as previously described. This assay was performed in RPMI without phenol red (Gibco, Melbourne). The RPMI was supplemented with 5 % heat-inactivated FCS. These plates were incubated for 48 hours at 37^0C after which they were frozen and thawed twice. The cell lysates were then tested for superoxide dismutase activity. The method followed for this assay was based on the principle of the inhibition of pyrogallol autoxidation by SOD as originally described by Marklund and Marklund (1974). The experimental method followed was similar to that described by Minami and Yoshikawa (1979) and Wei *et al.*, (1991) with the reaction volume being scaled down to 140 µl so the assays could be performed in flat-bottomed 96 well Linbro microtitre trays. To differentiate the predominant type of SOD produced in these assays,10 µl of 0.3 M sodium cyanide was included in repeat assays. CuZn SODs are inhibited by cyanides whereas Mn and Fe SODs are not affected (Marklund and Marklund, 1974). Negative controls were RPMI plus FCS alone and a Sigma (St. Louis) standard SOD (S 8254) was used. The plates were read on a Dyna-tech automated plate reader at 540 nm. The unknown values were calculated from an exponentially transformed standard curve.

Detection of Reactive nitrogen intermediates (RNIs) in the supernatants of TNF-treated and virus-infected L929 cells.

L929 cells were TNF-treated and infected as described above. This time however, the assays were performed in EMEM plus 5 % FCS and not RPMI. RPMI contains a large quantity of nitrate which interferes with the RNI measurements. EMEM contains no nitrates. After the 48 hour post infection culture period the L929 cell supernatants were removed for an RNI assay. The standards were diluted in EMEM plus FCS. RNIs were detected by an assay which utilises a copper-cadmium-zinc catalyst to convert nitrate to nitrite before exposure to Greiss reagent. This technique has been described in detail recently (Rockett *et al.*, 1992).

* The experiments performed for this chapter were all done at least twice unless otherwise indicated. Please note that (n =) refers to the number of animals or samples in an experimental or control group.

RESULTS.

The antiviral effects of TNF can be enhanced directly *in vitro*.

Figures 7.1 a) and 7.1 b) show the titres of HSV-1 and vaccinia virus respectively in L929 cells that had been treated with TNF ± Mab 32 twenty-four hours before infection. Both viruses were used at a MOI equal to 2.0. For the HSV-1 infected cells 10 ng TNF alone did not significantly inhibit the growth of the virus whereas at higher TNF doses (100-400 ng) there was a significant antiviral effect. The complex of TNF with Mab 32 showed significant enhancement of HSV-1 growth inhibition at the TNF doses of 10 and 200 ng. This was particularly apparent at the TNF dose of 10 ng because this 10 ng TNF dose alone did not elicit a significant effect. Pre-treatment of L929 cells with Mab 32 alone (100 ng) did not significantly effect the HSV-1 titre compared to the PBS control. In cells pre-treated with TNF ± Mab 32 and infected with vaccinia virus again the 10 ng TNF alone treatment had no significant antiviral effect whereas TNF alone doses of 100 and 400 ng were significantly antiviral. The 10 ng TNF plus Mab 32 treatment caused a significant decrease in vaccinia titre when compared to the 10 ng TNF alone treatment. At no other TNF dose tested was a significant enhancement of antiviral effect seen with Mab 32.

It is important to note that at no TNF dose tested, whether with or without Mab 32, was there an antiviral state induced in HeLa cells to HSV-1 (data not shown). As described in chapter 6 both L929 cells and HeLa cells showed enhanced TNF binding and internalisation in the presence of Mab 32.

TNF pre-treatment of L929 cells enhances HSV-1 induced cytopathogenicity.

L929 cells that were treated with TNF ± Mab 32 as described above but infected 24 hours later with a 20 times lower dose of HSV-1 (MOI = 0.1) showed varying degrees of microscopically visible cytopathic effect. If the L929 cells had been pre-treated with 400 ng TNF either alone or in complex with Mab 32 and then mock-infected 24 hours later there were no visible signs of cytopathic effect (Fig. 7.2 a and Fig. 7.2 b). However, if following 400 ng TNF plus Mab 32 pre-treatment the cells were infected with HSV-1 (MOI = 0.1) there appeared very obvious and widespread cytopathogenicity (Fig. 7.2 d) which was much more pronounced than that found if the cells had been pre-treated with 400 ng TNF alone (Fig. 7.2 c). Cells which had been treated with PBS 24 hours before infection with the lower dose of HSV-1 displayed no general cytopathogenicity (see Fig. 7.5 c).

The presence of Mab 32 with TNF *in vitro* enhances quantitative L929 cell death upon HSV-1 infection.

L929 cells that had been exposed to varying TNF concentrations with or without Mab 32 and either mock-infected or challenged with HSV-1 (MOI = 0.1) 24 hours later were stained with MTT to assess the extent of cell death (Fig. 7.3 a and 7.3 b). In the mock-infected L929 cells there were no significant differences in the amount of cell death between the TNF alone and the TNF plus Mab 32 treatments. In cells that had been treated with TNF the same way as above but infected with HSV-1 there was significantly greater cell death induced at every dose when TNF in complex with Mab 32 was compared to the TNF alone treatments. This degree of cell death in TNF plus Mab 32 treated cells was also greater than that found for cells which had not been pre-exposed to TNF (ie, PBS treated) and subsequently infected with HSV-1.

Possible mechanisms of enhanced cytopathogenicity in TNF plus Mab 32 pre-treated and HSV-1 infected L929 cells.

To elucidate the mechanism of enhanced cytotoxicity in TNF plus Mab 32 treated L929 cells on HSV-1 infection both the cells and cell supernatants were examined. Fig. 7.4 shows photographs of cellular DNA extracted from TNF ± Mab 32 treated and HSV-1 or mock-infected L929 cells. No detectable changes in DNA profile could be seen between the treatments at the 4 hours post-infection stage and for every treatment the DNA was largely intact. At 48 hours post-infection, even though there was demonstrable CPE in the relevant treatments, there was again no difference in the appearance of the DNA between treatments and again the DNA was generally intact. However at this 48 hours post-infection time point some smearing of the DNA was observed in most treatments which indicates the onset of cellular necrosis. No treatment at either time point displayed DNA profiles indicative of apoptosis (ie, "laddering" of DNA due to nuclease digestion at approximately 200 base pair intervals).

Table 7.1 shows a comparison of the lysis of L929 target cells by supernatants from TNF pre-treated and either mock-infected or HSV-1 infected L929 cells. This study showed that at both 24 and 48 hours there was no enhanced cytotoxicity in the supernatants from TNF plus Mab 32 pre-treated and HSV-1 infected cells when compared to controls that had been TNF plus Mab 32 treated and mock-infected. There was generally more cytotoxicity seen in the supernatants of TNF plus Mab 32 pre-treated cells when compared to supernatants from cells that had been pre-treated with TNF alone, except at the 48 hour time point for HSV-1 infected cells.

TABLE 7.1 Specific lysis of ^{51}Cr labelled L929 cells by U.V. irradiated supernatants from TNF treated and mock or HSV-1 (MOI = 0.1) infected L929 cells.

	24 Hours	**48 Hours**
Treatment	**Mock infected**	
No hTNF	9.4 % ± 0.9 %	16.5 % ± 1.1 %
400 ng hTNF	5.0 % ± 0.8 %	17.6 % ± 1.2 %
400 ng hTNF + Mab 32	15.4 % ± 1.4 %	27.5 % ± 5.4 %
	HSV-1 (MOI= 0.1)	
HSV-1 alone	1.8 % ± 0.2 %	13.4 % ± 0.7 %
400 ng hTNF + HSV-1	5.4 % ± 0.7 %	18.5 % ± 0.5 %
400 ng hTNF + Mab 32 + HSV-1	11.7 % ± 1.8 %	16.5 % ± 1.4 %

Supernatants from the above-described TNF ± Mab 32 treated and mock/HSV-1-infected cells were also screened for the presence of murine TNF and murine IL-1. Neither TNF nor IL-1 could be detected in the supernatants from L929 cells treated in this way (data not shown).

Possible involvement of free-radicals in HSV-1-induced cytotoxicity evident in TNF plus Mab 32 treated cells.

To investigate the possible role of free-radicals in this phenomenon butylated hydoxyanisole (BHA), a resonance stabilised free radical scavenger (Cowden *et al.*, 1985), was tested in this *in vitro* model. Fig. 7.5 (a] and b]) shows photographs taken of L929 cells which had been treated with TNF plus Mab 32 and then infected with

HSV-1 (MOI = 0.1). After infection of the cells, BHA (100 μmoles) was included in the fresh culture media of the relevant wells (Fig. 7.5 b). This treatment drastically reduced the general cytopathic effect (CPE) compared to non-BHA treated cultures (Fig.7.5 a). In cells which had been treated with PBS before infection and not exposed to BHA very little CPE was evident (Fig. 7.5 c]). In similarly treated cells that had BHA included in the culture medium after HSV-1 infection, however, CPE was evident (Fig. 7.5 d]). This is an opposite finding to that observed for cells that had been exposed to TNF plus Mab 32 before infection.

Correlation of cytopathic effect (CPE) with HSV-1 growth *in vitro*.

As stated earlier TNF can be directly antiviral for HSV-1 (MOI = 2.0) infected cells and furthermore Mab 32 can enhance these *in vitro* antiviral effects (Fig. 7.1 a]). This was also found if the TNF ± Mab 32 treated cells were infected with HSV-1 at a MOI equal to 0.1. Fig. 7.6 shows the comparison of *in vitro* HSV-1 growth in L929 cells that had been treated with TNF either alone or in complex with Mab 32 and exposed to BHA either before or after HSV-1 (MOI = 0.1) infection. There were no significant differences in the HSV-1 growth between PBS, TNF alone or TNF plus Mab 32 pre-treated cells that had either been exposed to BHA before infection or not exposed to BHA at all. If BHA was included in the cultures after the infection of the L929 cells dramatic increases in HSV-1 growth were seen for the PBS, TNF and TNF plus Mab 32 treatments. The virus growth in cells that were exposed to PBS before infection was significantly greater compared to both of the TNF pre-treatments and there was no significant difference between the TNF alone and TNF plus Mab 32 treatments. This suggests that while a BHA sensitive factor was important TNF may also activate other antiviral effects in these cells. Alternatively, the dose of BHA used

might not have been sufficient to completely nullify the action of TNF. For both the non-BHA treated controls and the cells exposed to BHA before infection the TNF alone treatments significantly inhibited the growth of HSV-1 compared to the respective PBS treatments. There was also some noticeable enhancement of this effect if TNF was in complex with Mab 32.

A titration of BHA-induced effects on HSV-1 growth in both PBS and 400 ng TNF alone treated L929 cells is described in Fig. 7.7. The maximum BHA effect was observed at the 100 and 200 μM doses. At every BHA dose tested there were significantly lower HSV-1 titres in the 400 ng TNF pretreated cells. This included the dose of 200 μmoles BHA (double what was routinely used) which supports the above statement that it is likely that other non-BHA sensitive antiviral factors were being induced by TNF in these cells.

Vaccinia virus induced cytopathic effect and growth in response to BHA treatment.

Fig. 7.8 shows photographs of L929 cells that were pre-treated with TNF ± Mab 32 exactly the same way as previously described but this time infected with vaccinia virus (MOI = 0.1). One hundred micromolar BHA was included in the relevant cultures after vaccinia infection. Fig. 7.8 a) shows cells that were pre-treated with TNF plus Mab 32 and not exposed to BHA after vaccinia infection. CPE was evident in these cultures although not as severe as that found for HSV-1 infected cells. With the inclusion of BHA in the cultures after infection the CPE effect was markedly reduced (Fig. 7.8 b]). In cells that had the control treatment of PBS before vaccinia infection (Figs. 7.8 c]) no obvious CPE was present due to virus alone. With the inclusion of BHA (Fig. 7.8 d]) in such cultures again no obvious vaccinia-induced CPE was evident, which is in contrast to what was found for HSV-1.

The growth of vaccinia virus in the above-described culture system with BHA is shown in Fig. 7.9. In the L929 cells that had been pre-treated with TNF ± Mab 32 a significant decrease in virus growth was observed compared to the PBS control both with and without BHA. The presence of BHA in the cultures after infection did not result in the dramatic increase of virus growth that was observed with HSV-1. In cells that had been pre-treated with PBS the growth of vaccinia was identical for BHA and non-BHA treated cultures. In the cells treated before infection with TNF alone there was a small but non-significant increase in growth due to BHA. Cells pre-treated with TNF plus Mab 32 showed a small increase in vaccinia virus growth in the presence of BHA compared to the other TNF (± BHA) treatments. As previously mentioned however in no case was there an increase in vaccinia virus growth due to BHA treatment similar in magnitude to that observed for HSV-1.

Superoxide Dismutase (SOD) activity in TNF ± Mab 32 treated and HSV-1 or Vaccinia virus infected L929 cells.

To gauge the comparative activity of free-oxygen radicals, particularly that of the superoxide radical (O_2^-), TNF treated and HSV-1 or vaccinia virus infected L929 cultures were measured for SOD. The findings of this experiment are presented in Fig. 7.10. The SOD activity was significantly greater for both the TNF alone and TNF plus Mab 32 pre-treatments in HSV-1 infected cultures compared to similarly pre-treated L929 cell cultures infected with the same dose of vaccinia virus. This was particularly apparent in the cells pre-treated with TNF plus Mab 32. Cells pre-treated with PBS also showed greater SOD activity with HSV-1 infection compared to vaccinia virus infection. There was no significant variation in SOD activity between PBS and TNF alone pretreated cultures which had been infected with HSV-1 and this was also true

for the PBS and TNF pretreatments in vaccinia virus infected cells. A small non-significant increase in SOD activity was seen with TNF plus Mab 32 treatment compared to the TNF alone treatment in vaccinia virus infected cells. The comparison of TNF alone and TNF plus Mab 32 treatments for HSV-1 infected cells showed approximately a 2 fold mean increase in SOD activity for the TNF plus Mab 32 treated cells. Cell cultures that had been pre-treated with PBS, TNF alone or TNF plus Mab 32 and mock-infected displayed no detectable SOD activity (data not shown).

To differentiate the type of SOD produced in these virus-infected cells sodium cyanide was included in repeat assays. For PBS, TNF alone and TNF plus Mab 32 pre-treated L929 cells infected with vaccinia virus or HSV-1 SOD activity was almost totally inhibited by the inclusion of sodium cyanide. This indicates that copper/zinc SOD is the class of this enzyme produced by HSV-1 and vaccinia virus infection under these experimental conditions (data not shown).

Reactive Nitrogen Intermediates (RNIs) in the control of HSV-1 growth *in vitro*.

To examine the possible role of RNIs as a factor in the regulation of HSV-1 growth by BHA, a specific nitric oxide synthetase (the enzyme which produces nitric oxide) inhibitor N^G-ethyl-L-arginine acetate (NELA) was tested in the established *in vitro* model. Fig. 7.11 shows that with PBS, TNF alone or TNF plus Mab 32 pre-treatment of L929 cells no significant increase in HSV-1 growth was seen in the presence of NELA added after infection suggesting no significant role for RNIs in the direct control of HSV-1 growth. The lack of a role for RNIs is supported by table 7.2 which shows no significant differences in the amount of RNI secreted from PBS or TNF ± Mab 32 treated cells subsequently infected with HSV-1 (MOI = 0.1) either with or without BHA present. In control cells that were mock-infected (data not shown)

there were no significant differences in RNI secretion seen when compared to the above described HSV-1 infected cells.

TABLE 7.2 Levels of Reactive Nitrogen Intermediates (RNI) in the supernatants of TNF treated L929 cell cultures infected with HSV-1 (MOI= 0.1) 24 hours later. BHA was added to relevant cultures.

	REACTIVE NITROGEN INTERMEDIATES (μM)		
	PBS	**400 ng TNF**	**400 ng TNF + Mab 32**
No BHA	5.53 ± 0.76	6.98 ± 1.99	6.38 ± 0.40
BHA (100 μmoles)	5.30 ± 0.50	7.35 ± 0.69	8.43 ± 1.36

*The above described data were the results of a single experiment.

The influence of cyclooxygenase/lipoxygenase inhibitors on HSV-1 growth *in vitro*.

BHA as well as being a free-radical scavenger can also inhibit part of the arachidonic acid cascade. To test whether this was involved in the observed effects of BHA on HSV-1 growth 3 inhibitors of the arachidonic acid cascade (via cyclooxygenase, lipoxygenase pathway inhibition) were tested. These inhibitors were 1) ibuprofen (cyclooxygenase pathway; Dunn & Chuluyan, 1992), 2) BWA 137C (lipoxygenase pathway; Bhattacherjee *et al.*, 1988) and 3) BW 755C (cyclooxygenase and lipoxygenase pathways; Bhattacherjee *et al.*, 1988; Dunn & Chuluyan, 1992). The

dose range tried for the inhibitors did not cause any visible cell toxicity with the exception of the 100 μmole BWA 137C treatment. Fig. 7.12 shows the growth of HSV-1 in L929 cells treated with the 3 mentioned inhibitors. For ibuprofen and BWA 137C there was no significant effect on viral growth compared to the organic solvent alone control (the organic solvent control did not differ from the PBS control). BW 755C however showed a dose related inhibition of HSV-1 growth, suggesting that its effects are different to those of BHA as the presence of BHA increased HSV-1 growth under these experimental conditions.

SUMMARY.

Mab 32 was shown to be capable of enhancing the direct antiviral effects of TNF in L929 cells for both HSV-1 and vaccinia virus. This antiviral effect for HSV-1 was characterised by enhanced CPE in TNF pretreated cells which was further potentiated if Mab 32 had been present with TNF. Further study of this phenomenon showed, through the use of an antioxidant (BHA), that free oxygen radicals were possibly responsible for this CPE effect, which when reversed by BHA resulted in dramatic increases in HSV-1 growth. Direct free radical production appears to be important to the control of HSV-1 growth *in vitro*, and prior exposure of such cells to TNF, particularly with Mab 32, sensitised the cells to damage by free radicals induced by HSV-1 infection. Vaccinia virus did not exhibit the same degree of CPE in TNF exposed L929 cells, and did not show a significant increase in growth on BHA exposure. These comparative effects observed for HSV-1 versus vaccinia virus suggests an interesting dichotomy in the response of viruses to free radical induction. This difference was further supported by SOD studies which showed that superoxide activity was greater in HSV-1 infected cells, particularly if they had been pre-exposed to TNF ± Mab 32.

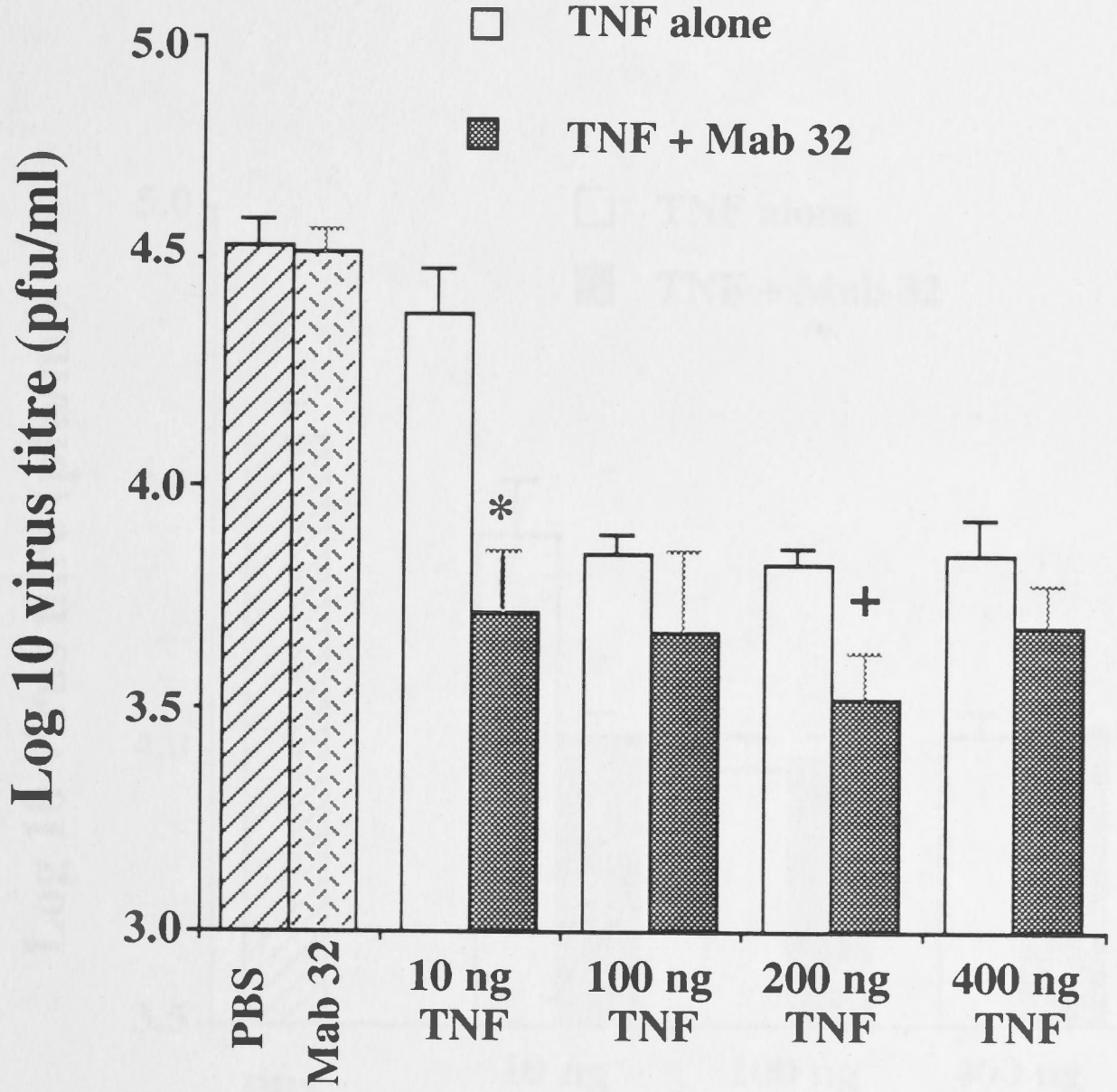


Figure 7.1 a) Mean growth of HSV-1 (MOI = 2.0) in TNF ± Mab 32 pretreated L929 cells. (n = 3) for each treatment.

* p = 0.017 and + p = 0.05 versus the TNF alone treatments at the same dose.

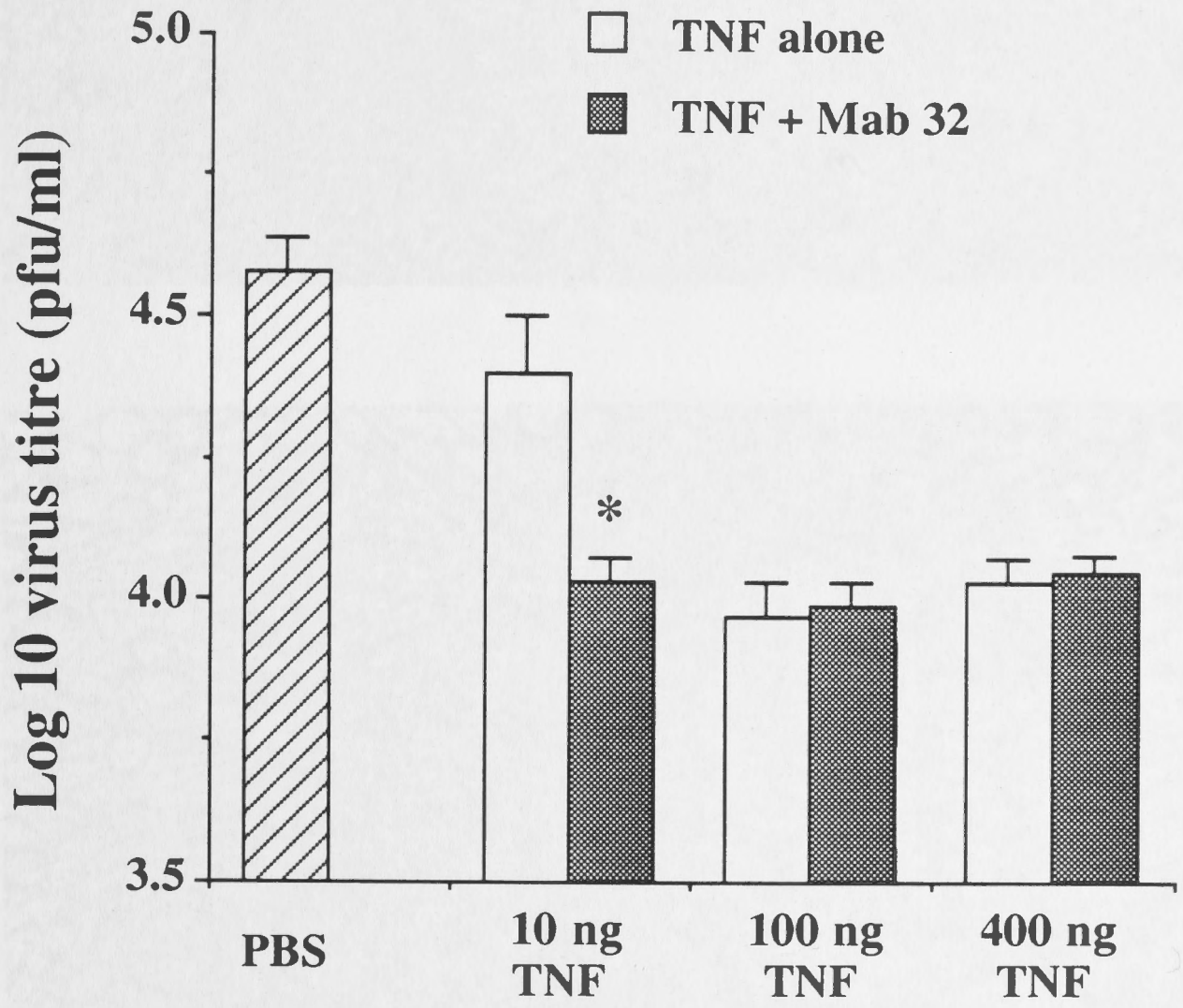
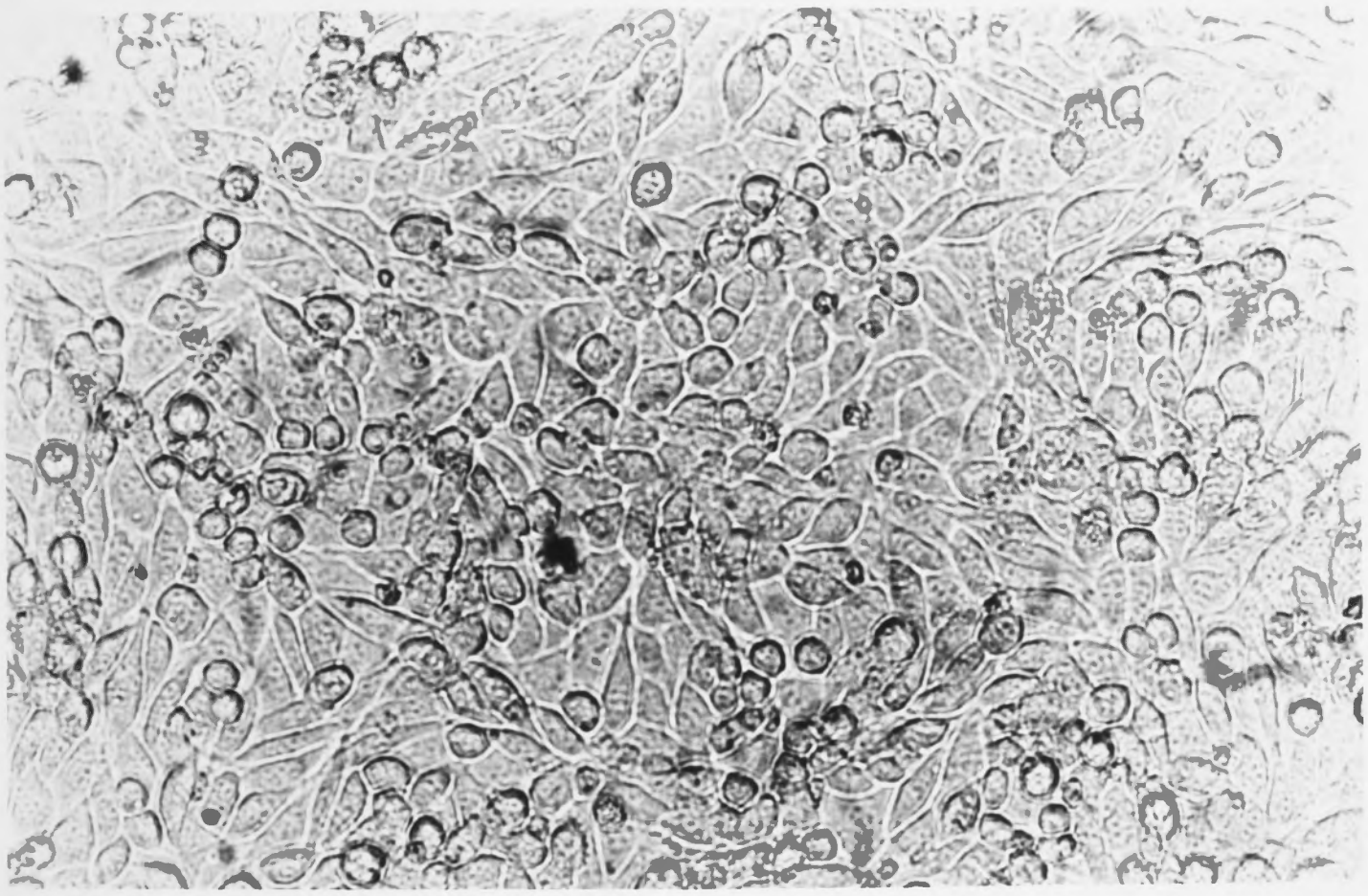


Figure 7.1 b) Mean growth of Vaccinia virus (MOI = 2.0) in TNF ± Mab32 pretreated L929 cells. (n = 3) for each treatment.

* p = 0.028 versus the 10 ng TNF alone treatment.

a)



b)

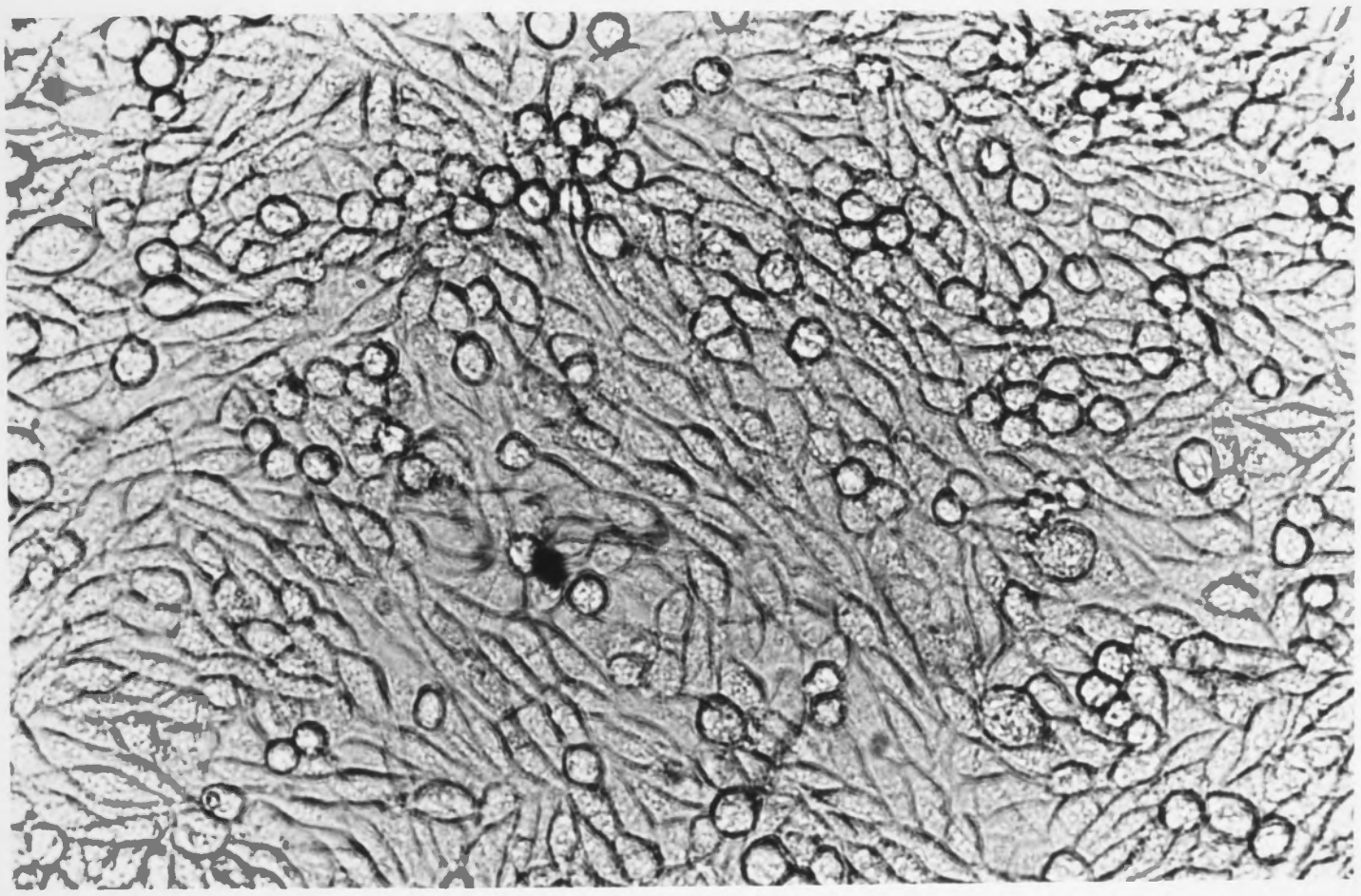
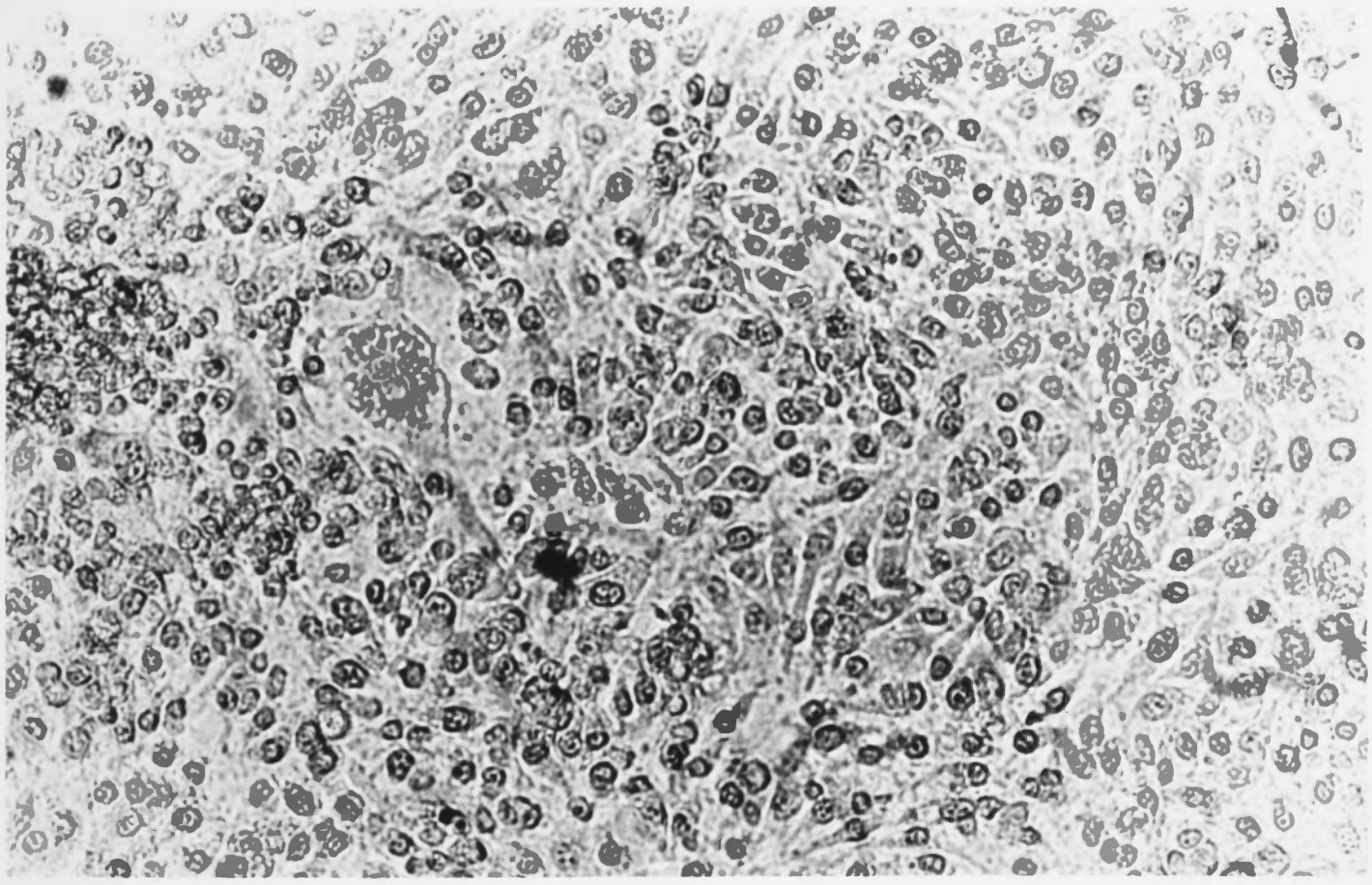


Fig. 7.2 Cytopathic effect observed in L929 cells that had been,

a) pre-treated with 400 ng TNF alone and mock-infected 24 hours later;

b) pre-treated with 400 ng TNF plus Mab 32 and mock-infected 24 hours later. (Magnification = X 400).

c)



d)

Fig. 7.2 (continued) Cytopathic effect observed in L929 cells that had been, **c)** pre-treated with 400 ng TNF alone and HSV-1-infected (MOI = 0.1) 24 hours later; **d)** pre-treated with 400 ng TNF plus Mab 32 and HSV-1-infected (MOI =0.1) 24 hours later. (Magnification = X 400).

a)

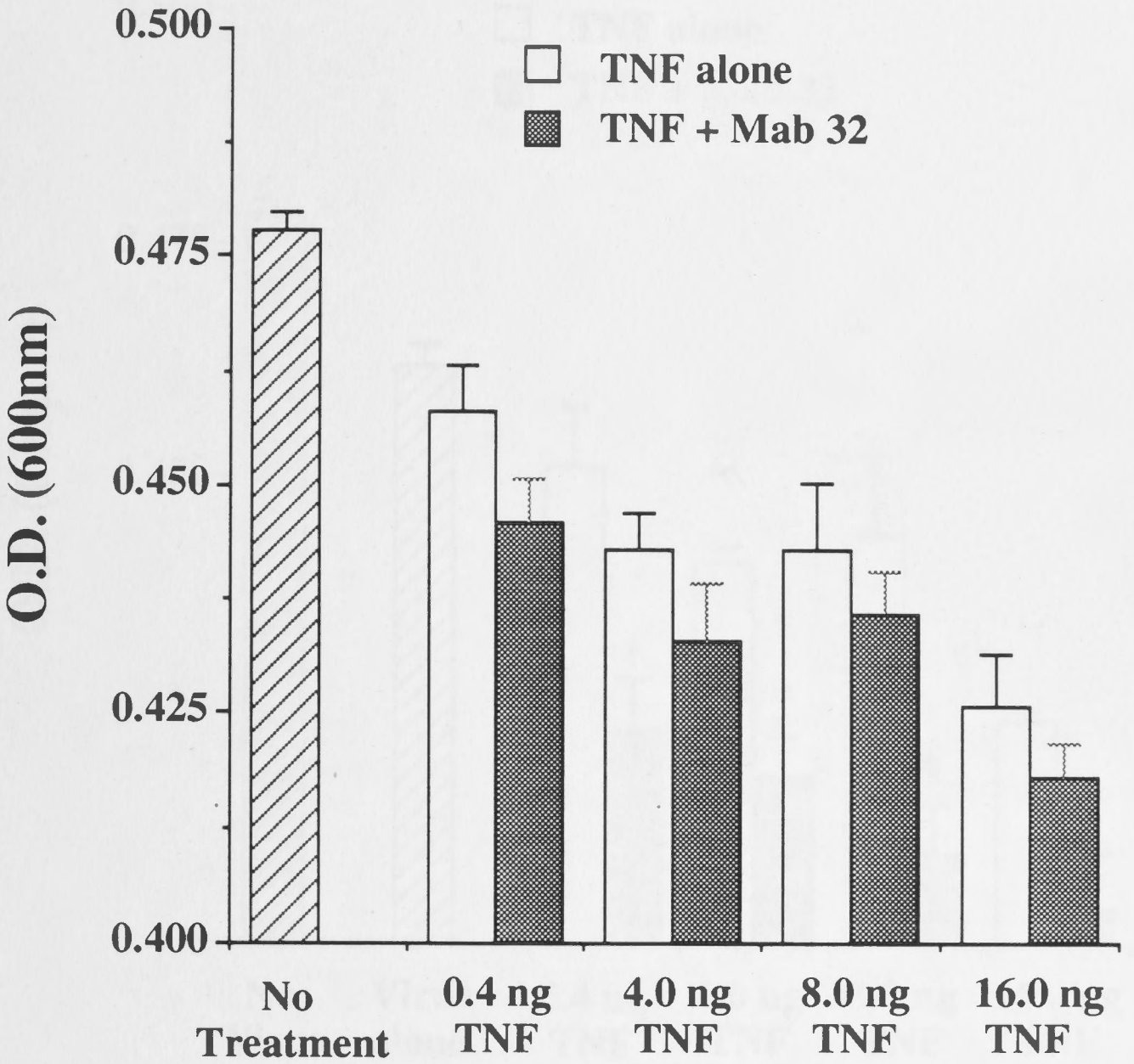


Figure 7.3 a) Cell death estimated by MTT staining for L929 cells pretreated with TNF ± Mab 32 and mock-infected 24 hours later. (n = 6) for each treatment.

TNF alone versus TNF + Mab 32 treatments were not significantly different at any TNF dose tested ($0.50 > p > 0.110$).

*The above described data were the results of a single experiment.

b)

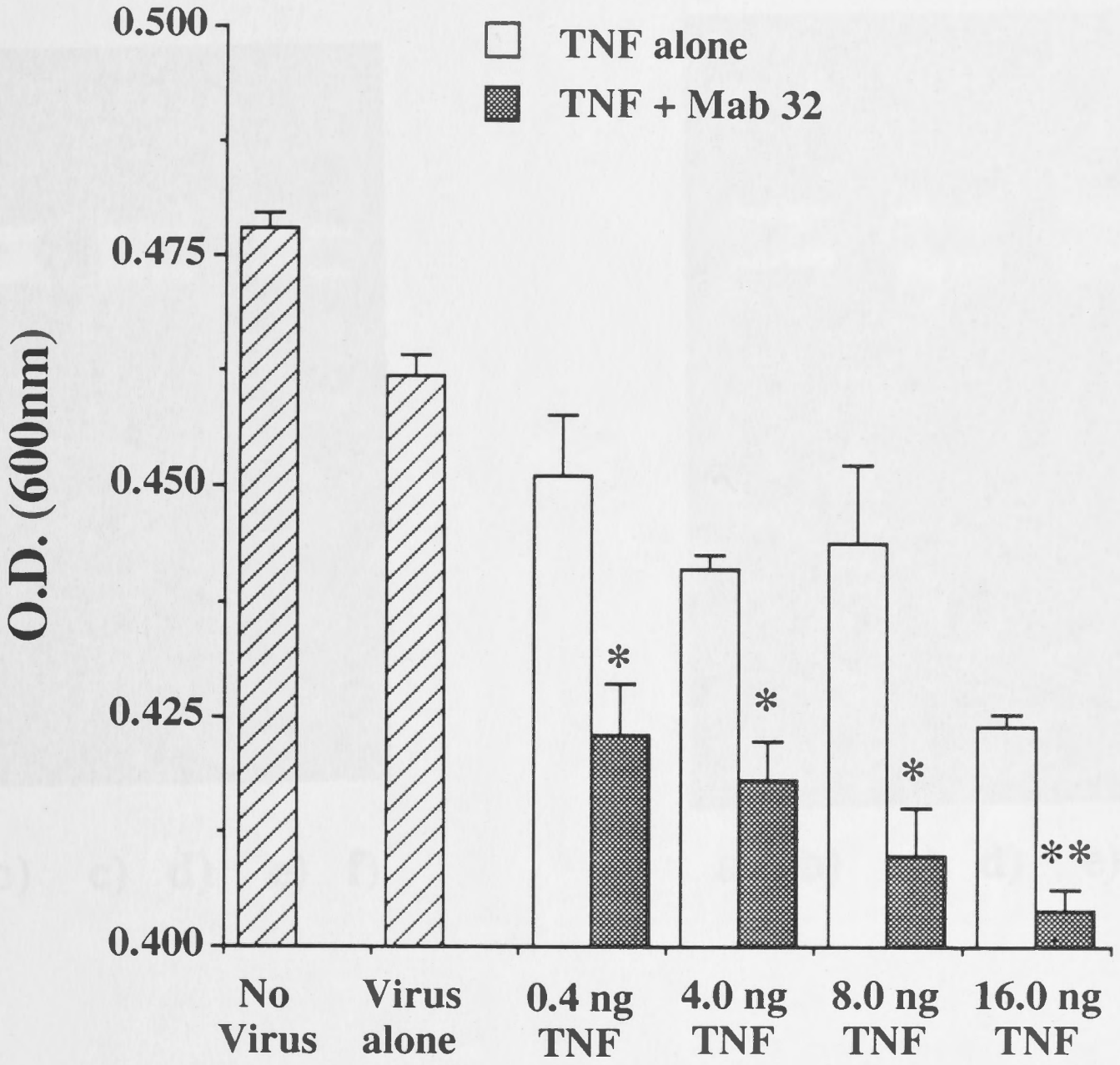
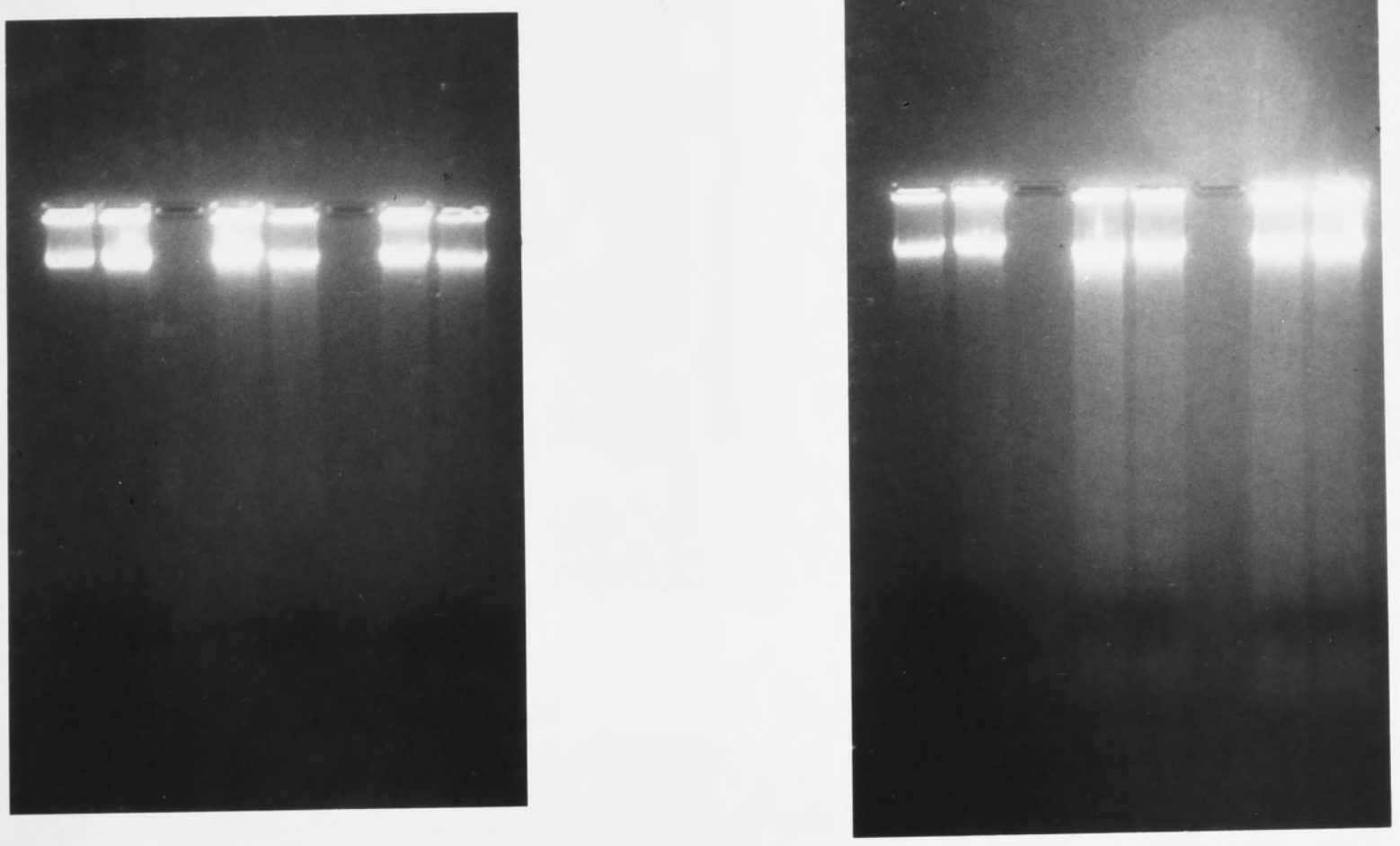


Figure 7.3 b) Cell death estimated by MTT staining for L929 cells pretreated with TNF ± Mab 32 and HSV-1-infected (MOI = 0.1) 24 hours later. (n = 6) for each treatment. TNF alone versus TNF + Mab 32 treatments were significantly different in at every TNF dose tested; * $0.010 > p \geq 0.001$; ** $p < 0.001$.

4 hours post-infection

48 hours post infection



a) b) c) d) e) f) **a) b) c) d) e) f)**

Fig. 7.4 DNA profile of L929 cells pre-treated with TNF (with and without Mab 32) and either infected with HSV-1 (MOI = 0.1) or mock-infected. Treatments were as follows;

a) Not treated/not infected,

b) HSV-1 infection alone,

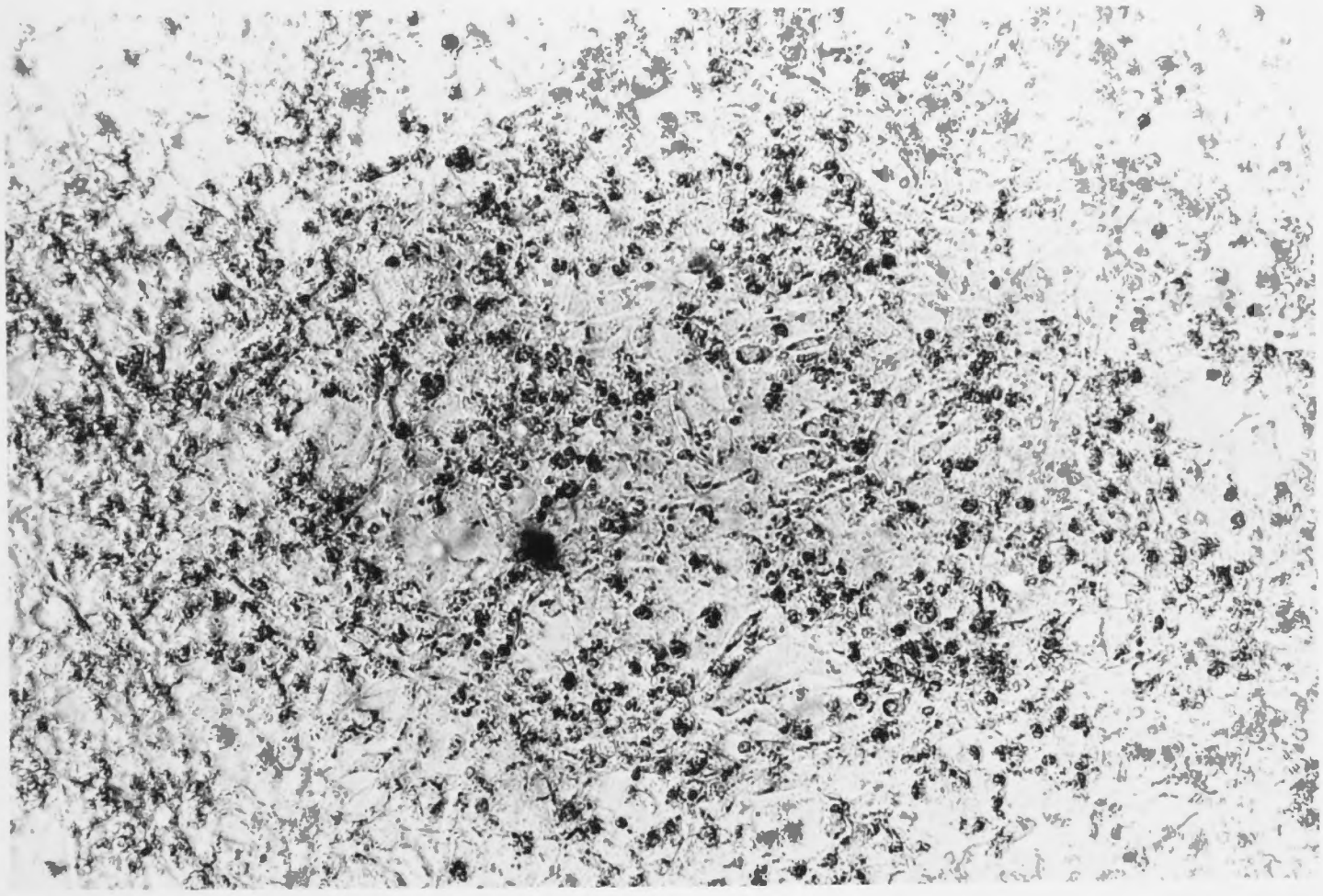
c) 400 ng TNF alone,

d) 400 ng TNF plus Mab 32 alone,

e) 400 ng TNF plus HSV-1 infection,

f) 400 ng TNF plus Mab 32 plus HSV-1 infection.

a)



b)

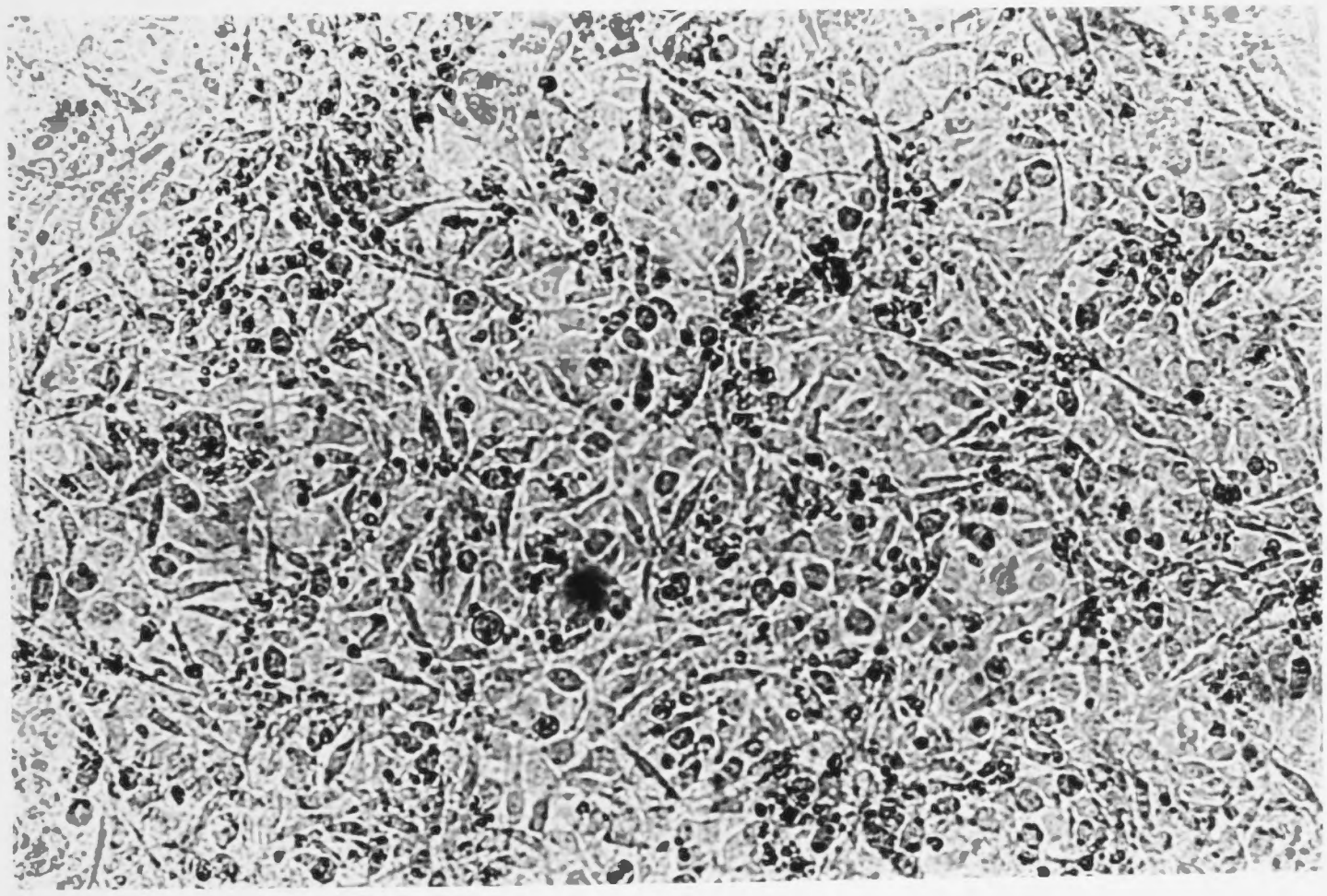
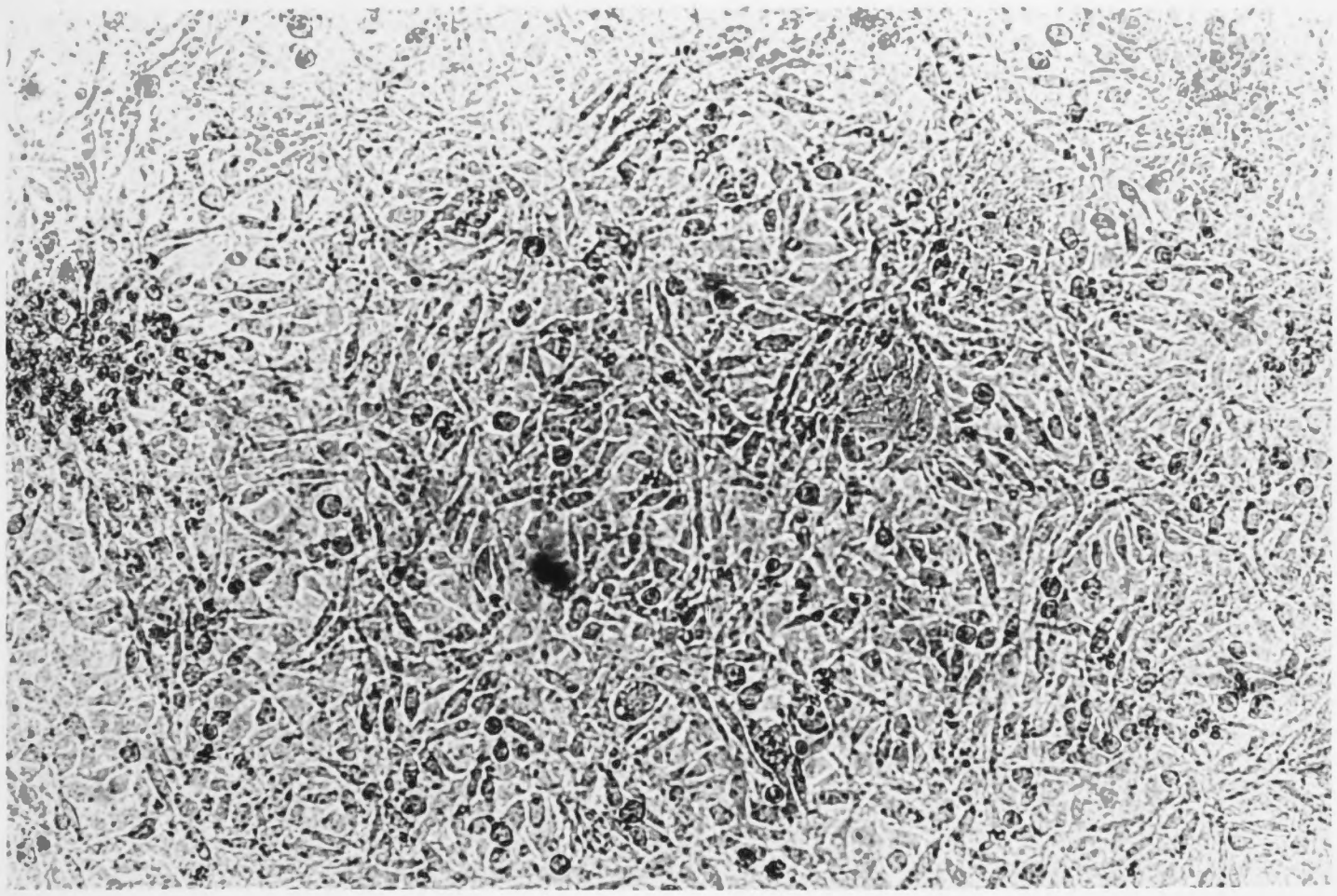


Fig. 7.5 Cytopathic effect observed in TNF plus Mab 32 pre-treated L929 cells that were infected 24 hours later with HSV-1 (MOI = 0.1) and **a)** not exposed to BHA, or **b)** Exposed to 100 μM/ml BHA. (Magnification = X 400).

c)



d)

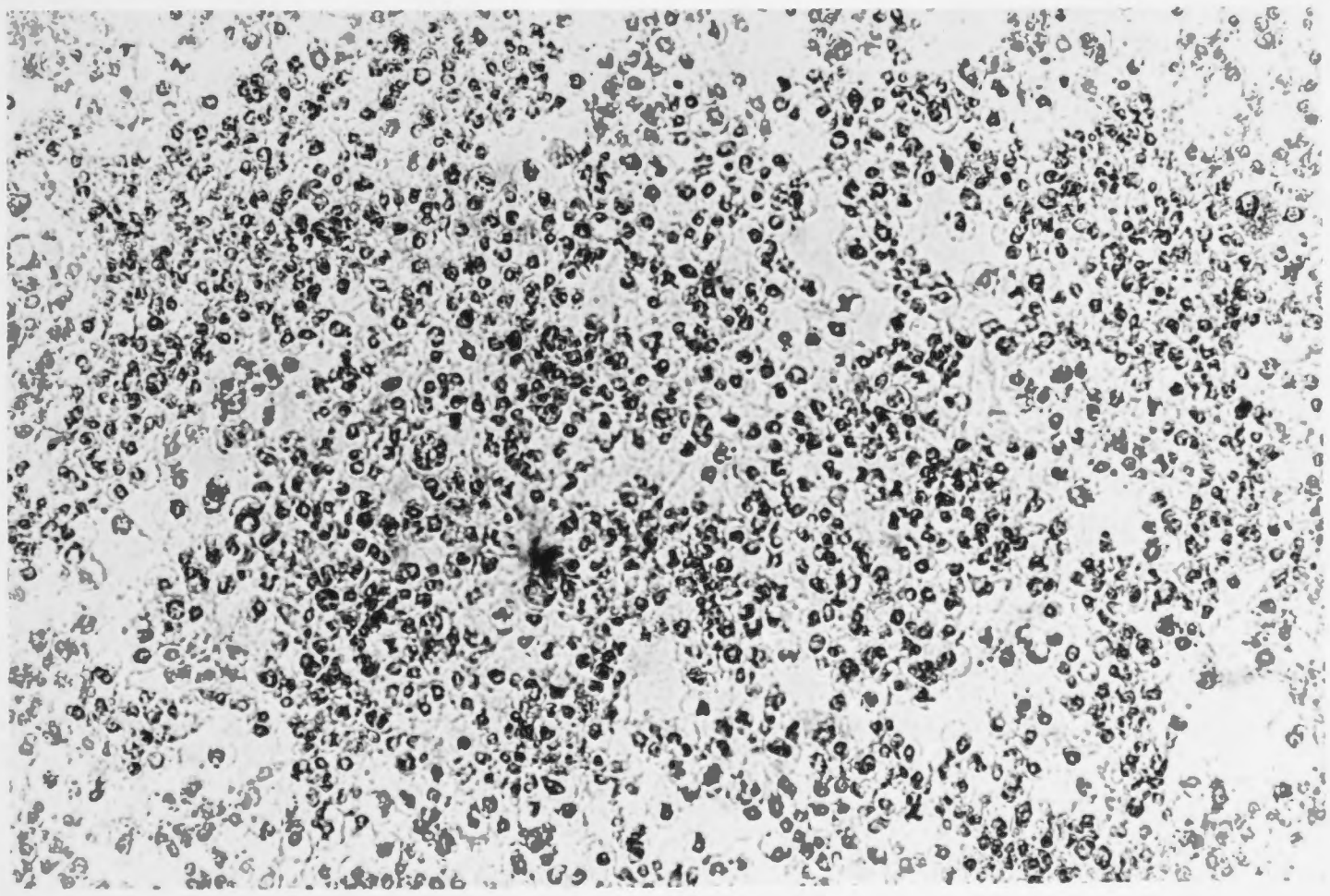


Fig. 7.5 (continued) Cytopathic effect observed in PBS pre-treated L929 cells that were infected 24 hours later with HSV-1 (MOI = 0.1) and **c)** not exposed to BHA, or **d)** Exposed to 100 μM/ml BHA. (Magnification = X 400).

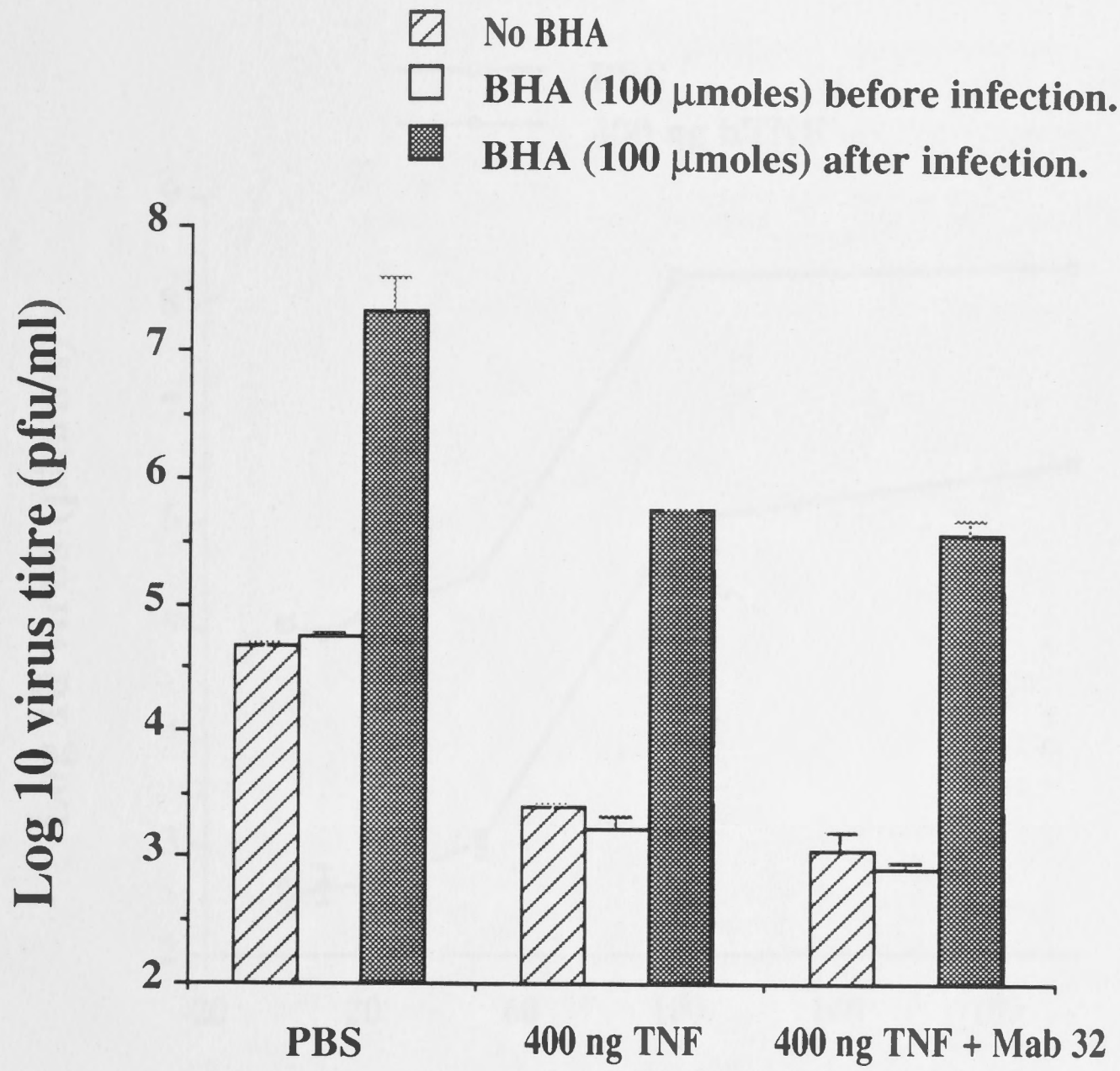


Figure 7.6 Mean growth of HSV-1 (MOI = 0.1) in TNF ± Mab 32 pretreated L929 cells which were also treated with 100 μmoles of BHA either before or after infection.

There were no significant differences between any treatment when the non-BHA treated controls were compared to the cells exposed to BHA before infection, ($0.36 > p > 0.08$).

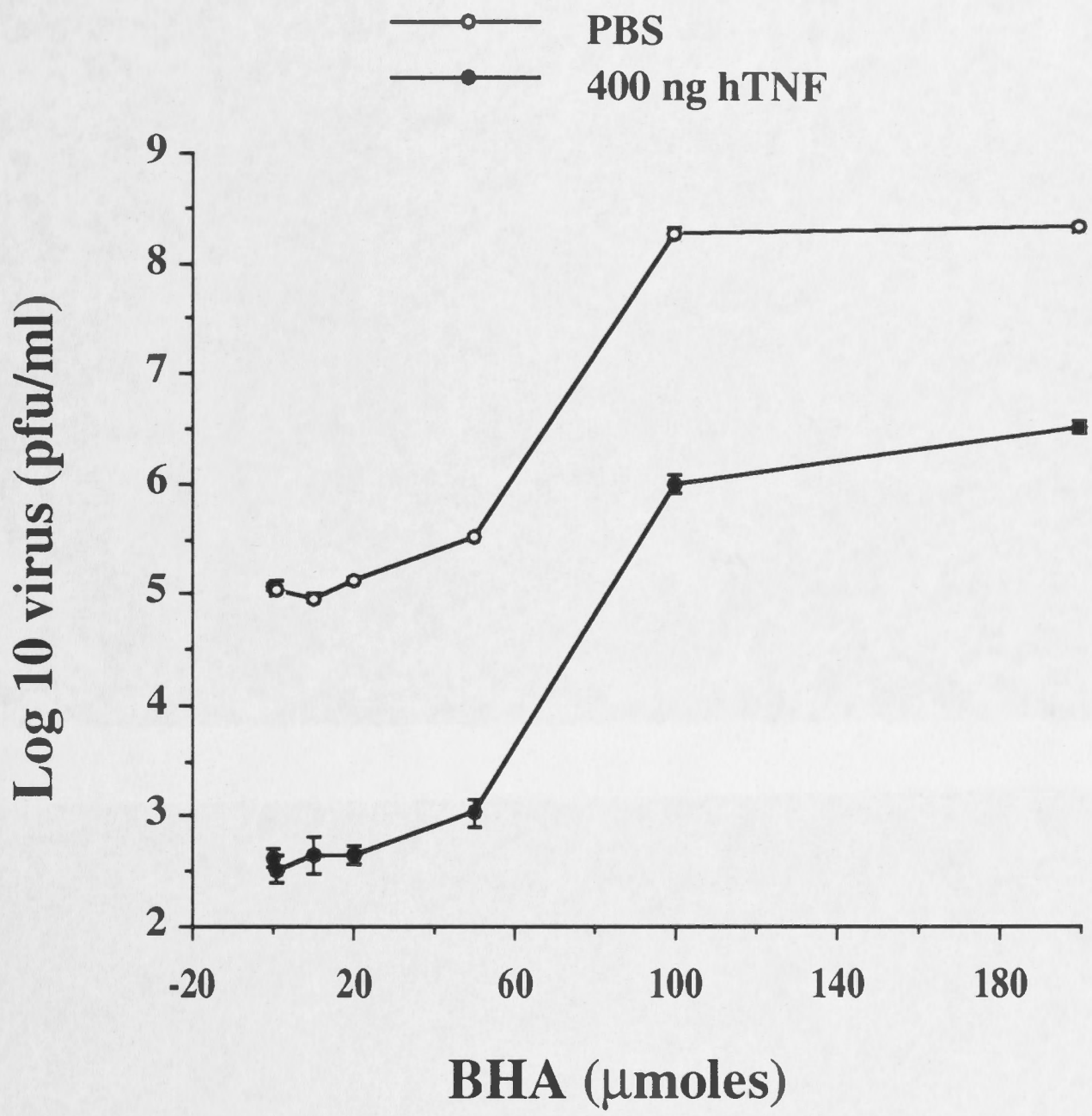
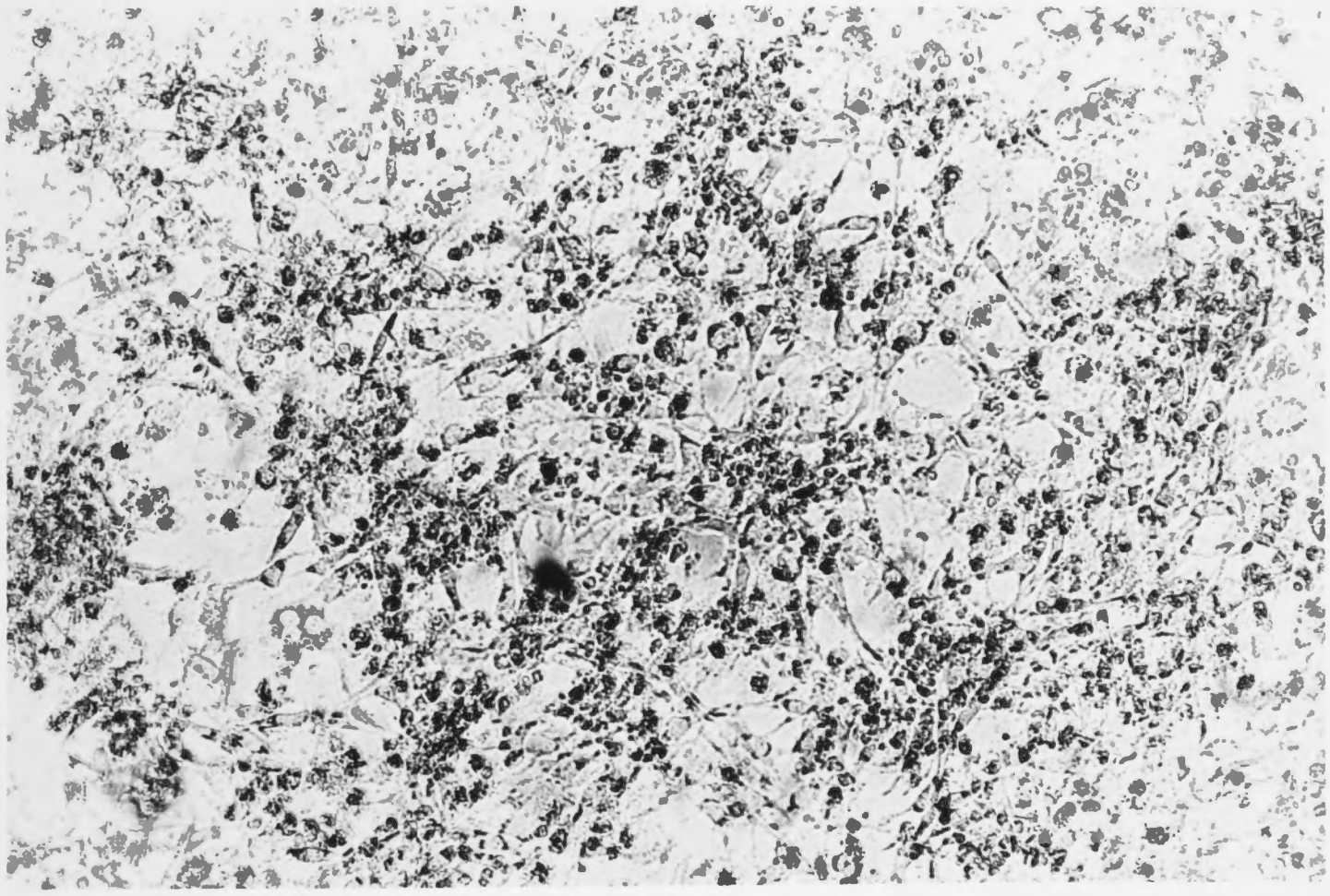


Figure 7.7 Titration of BHA (0-200 μmoles) effects when added after HSV-1 (MOI = 0.1) infection of L929 cells pretreated with PBS or 400 ng TNF.

a)



b)

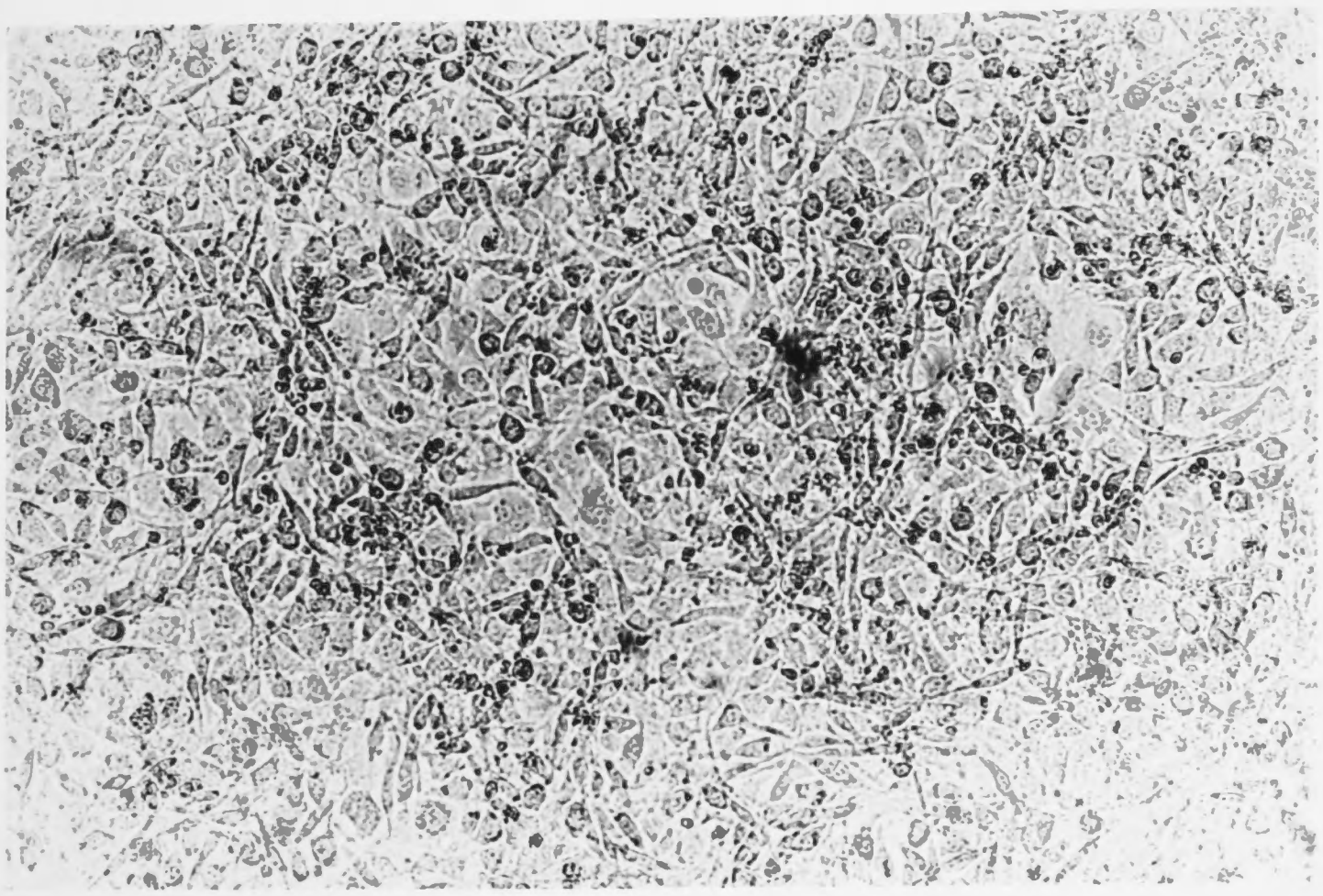
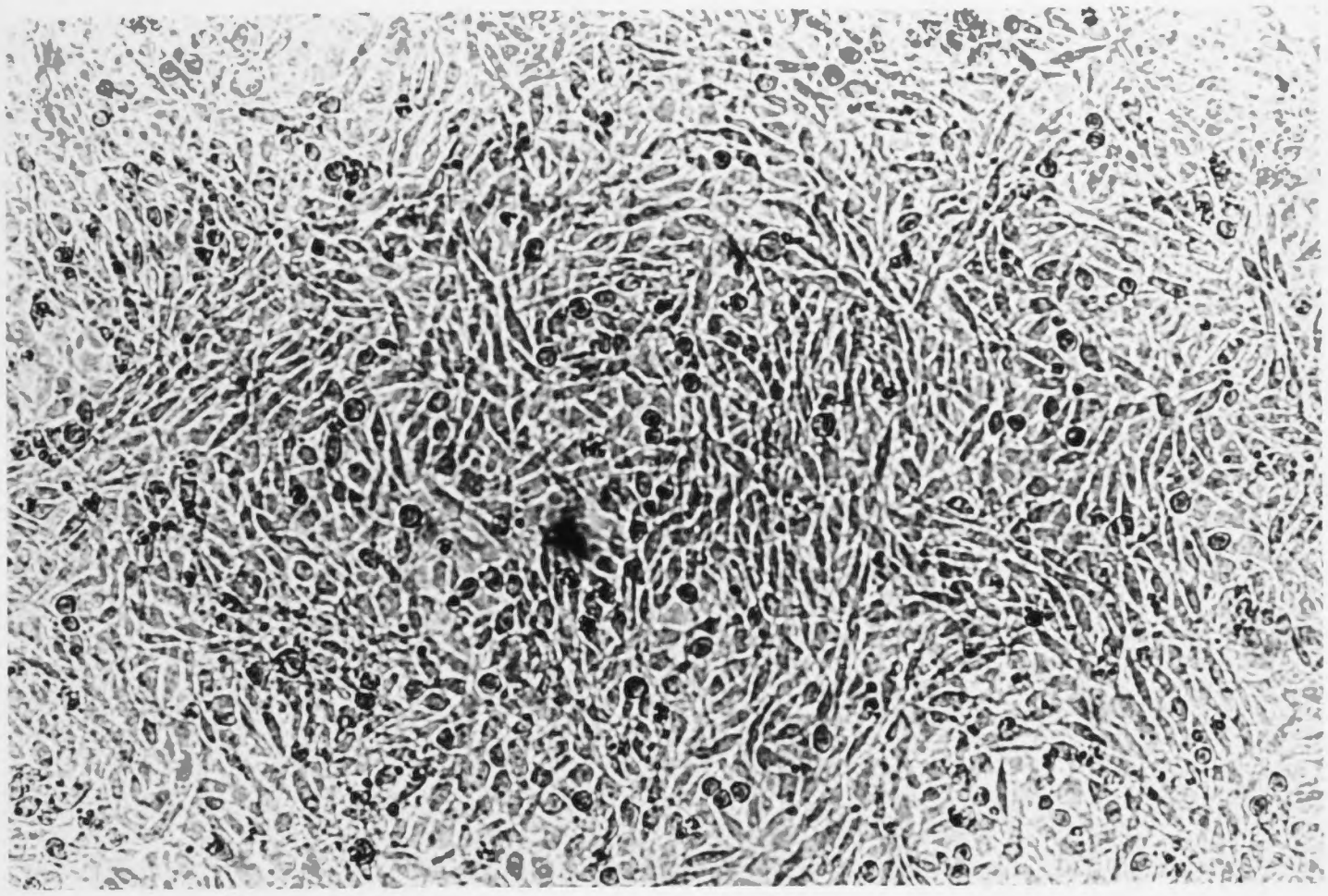


Fig. 7.8 Cytopathic effect observed in TNF plus Mab 32 pre-treated L929 cells that were infected 24 hours later with Vaccinia virus (MOI = 0.1) and **a)** not exposed to BHA, or **b)** Exposed to 100 µM/ml BHA. (Magnification = X 400).

c)



d)

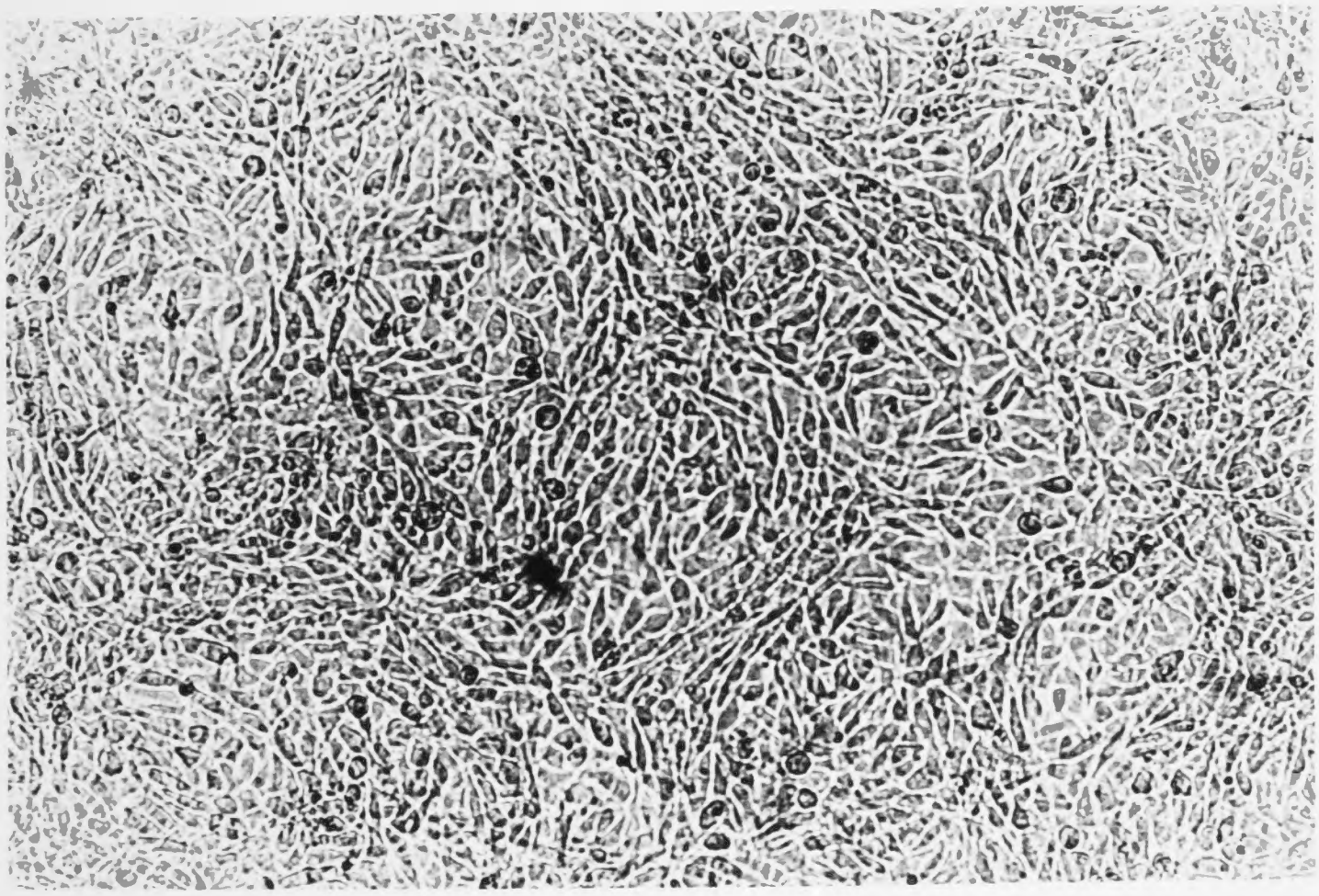


Fig. 7.8 (continued) Cytopathic effect observed in PBS pre-treated L929 cells that were infected 24 hours later with Vaccinia virus (MOI = 0.1) and **c)** not exposed to BHA, or **d)** Exposed to 100 μM/ml BHA. (Magnification = X 400).

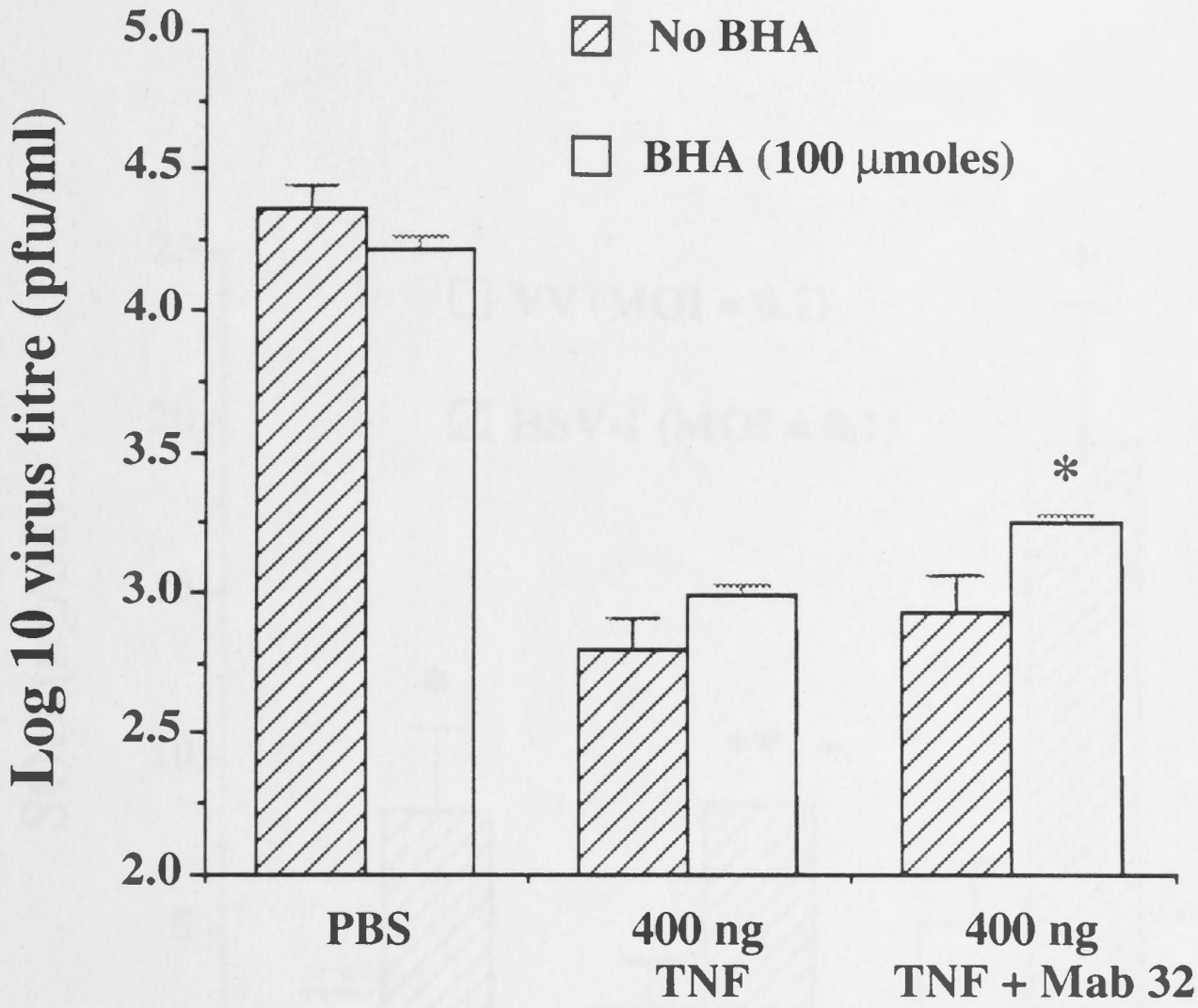


Figure 7.9 Mean growth of Vaccinia virus (MOI = 0.1) in TNF ± Mab 32 pretreated L929 cells which were also treated with 100 μmoles of BHA after infection.

* p = 0.08 versus TNF + Mab 32 pretreated cells not exposed to BHA after vaccinia virus infection.

For PBS and TNF alone pretreated cells there were no significant differences in virus titre between BHA exposed and BHA non-exposed cells ($0.23 > p > 0.15$).

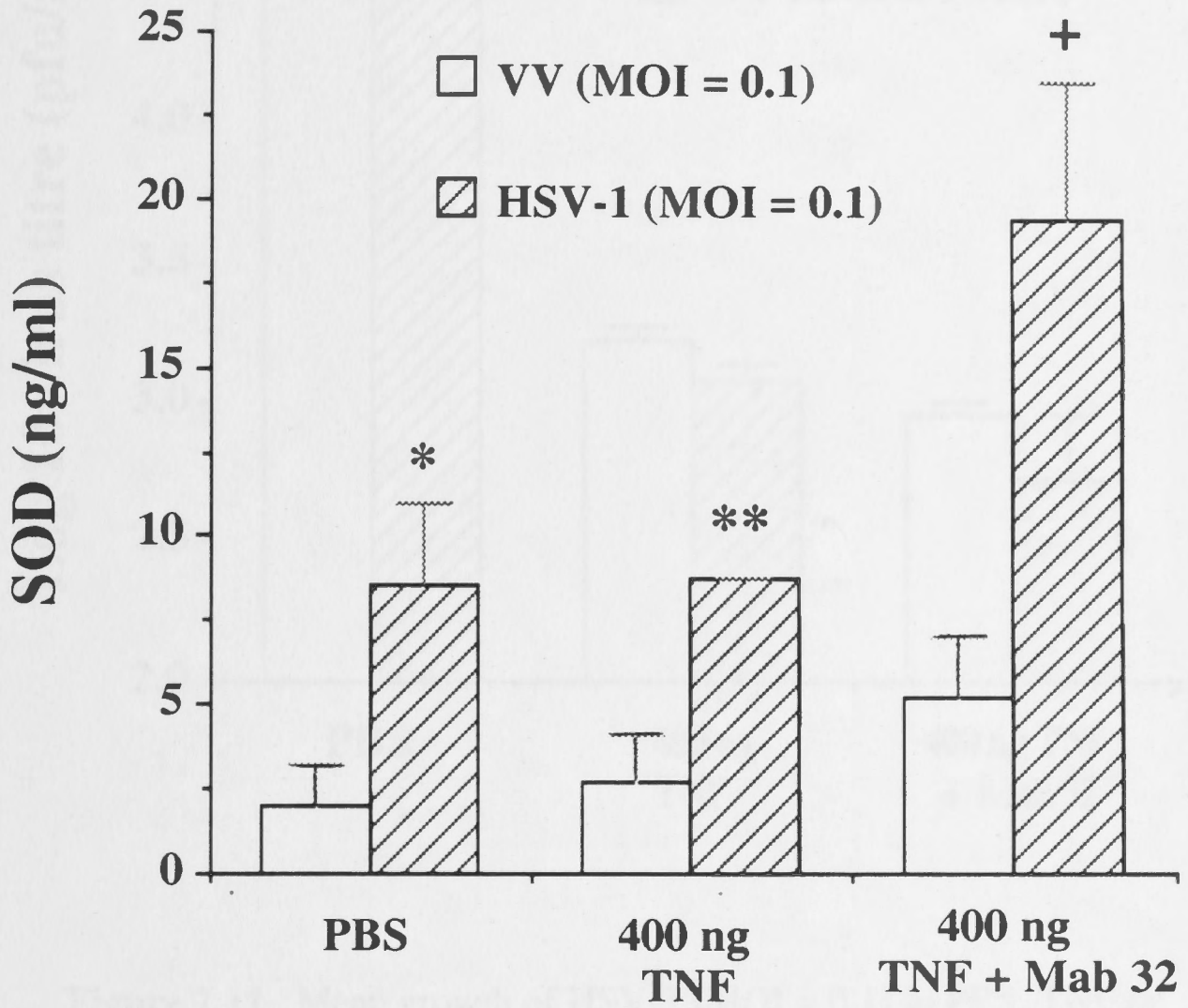


Figure 7.10 Mean Superoxide Dismutase (SOD) activity in L929 cell cultures which had been pretreated with PBS, TNF or TNF + Mab 32 and infected (MOI = 0.1) with either HSV-1 or Vaccinia virus. (n = 3) for each treatment.

* p = 0.077 versus PBS treated, vaccinia virus infected cells.

** p = 0.043 versus TNF treated, vaccinia virus infected cells.

+ p = 0.035 versus TNF + Mab 32 treated, vaccinia virus infected cells.

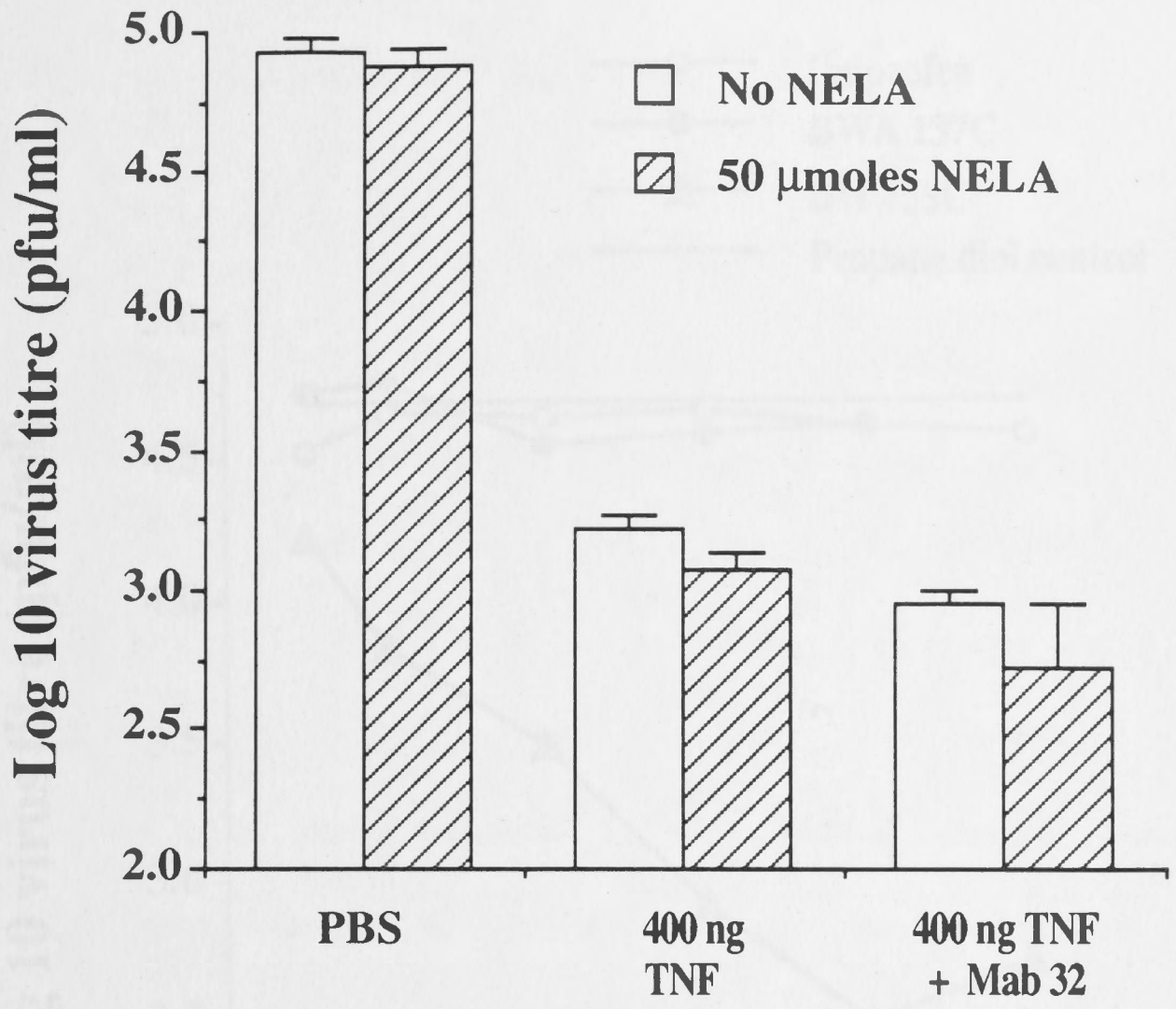


Figure 7.11 Mean growth of HSV-1 (MOI = 0.1) in PBS, TNF or TNF + Mab 32 pretreated L929 cells exposed to 50 μmoles N^{G}-ethyl-L-arginine acetate (NELA) after infection.
(n = 3) for each treatment.
There were no significant differences in virus titre for PBS, TNF or TNF + Mab 32 pretreated cells that had been exposed to NELA after infection compared to cells which had not been exposed to NELA,
($0.62 > p > 0.11$).

*The above described data were the results of a single experiment.

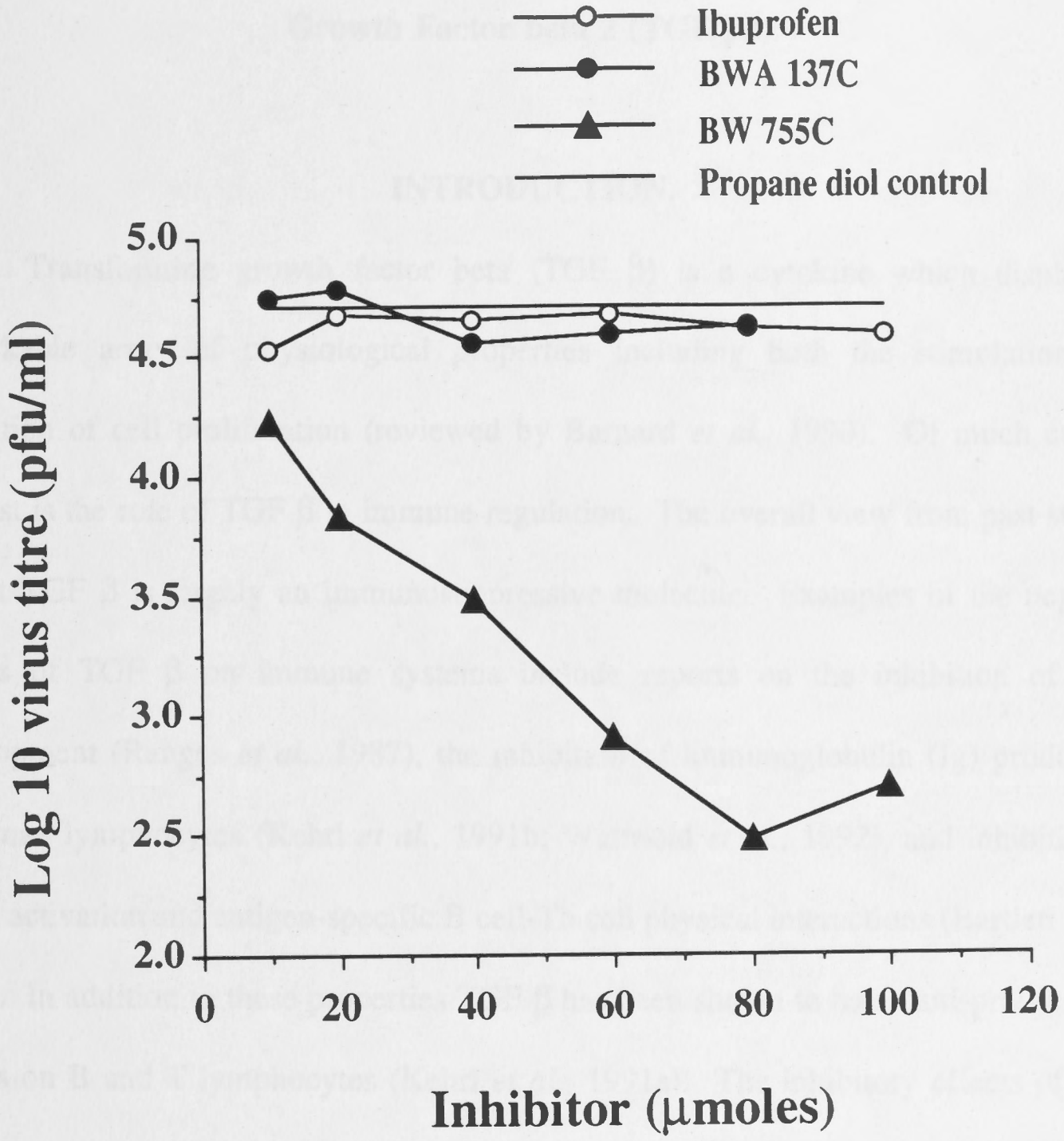


Figure 7.12 Growth of HSV-1 in L929 cells which had been exposed to between 10-100 μmoles of cyclooxygenase / lipoxygenase inhibitors for the duration of cell culture.

Chapter 8

The Immunobiology of a Vaccinia Virus Construct Encoding Transforming Growth Factor beta 2 (TGF β2).

INTRODUCTION.

Transforming growth factor beta (TGF β) is a cytokine which displays a remarkable array of physiological properties including both the stimulation and inhibition of cell proliferation (reviewed by Barnard *et al.*, 1990). Of much current interest is the role of TGF β in immune regulation. The overall view from past studies is that TGF β is largely an immunosuppressive molecule. Examples of the negative effects of TGF β on immune systems include reports on the inhibition of CTL development (Ranges *et al.*, 1987), the inhibition of immunoglobulin (Ig) production in human lymphocytes (Kehrl *et al.*, 1991b; Warmold *et al.*, 1992), and inhibition of B cell activation and antigen-specific B cell-Th cell physical interactions (Bartlett *et al.*, 1991). In addition to these properties TGF β has been shown to have anti-proliferative effects on B and T lymphocytes (Kehrl *et al.*, 1991a). The inhibitory effects of TGF β on immune function extend much further than the above examples both *in vitro* and *in vivo* and Palladino *et al.* (1989) gives a comprehensive summary of both TGF β mediated inhibitory effects as well as immune functions enhanced by TGF β. As documented in this review the immunosuppressive effects of TGF far outweigh the immuno-enhancing effects of this molecule. One interesting property of TGF both *in vitro* and *in vivo* is that it is capable of both augmenting and inhibiting TNF α production. There has been a study which has examined the effects of TGF β1 on immune function in a whole animal model (Strassmann *et al.*, 1991). In this study it was found that the administration of liposome associated TGF β1 to mice which had

been previously infected with *Corynebacterium parvum* reduced both the spleen size and the number of splenocytes compared to controls. As well as this there were inhibited DTH responses to *Listeria monocytogenes* in mice treated with the liposome-TGF β1 complex compared to control treated animals.

The aim of this chapter is to examine the role of TGF β2 in vaccinia virus infection using the recombinant vaccinia virus system which has been previously applied to the study of other cytokines (Ramshaw *et al.*, 1992).

MATERIALS and METHODS.

Construction of the recombinant vaccinia virus encoding murine TGF β_2.

The full length cDNA for murine TGF β_2, in a pBluescript II KS +/- vector (Stratagene), was generously provided by Dr. F. Denhez of the National Institute of Health, Maryland USA. The entire mTGF β_2 coding sequence was removed from the bluescript vector using Hinc II (Pharmacia) and Xba I (Pharmacia) endonucleases. The Xba I end of the gene was filled using the Klenow fragment of DNA polymerase I and the TGF gene then blunt-end ligated into the Hinc II site of the pBCB07 vaccinia expression vector (Boyle *et al.*, 1985; Andrew *et al.*, 1987). The inserted TGF β gene, which had it's orientation checked by restriction analysis, was directly downstream from the P7.5 vaccinia early/late promoter. This recombinant plasmid was used to transfect confluent 143 B cells which had been infected an hour earlier with VV-WR-L929 (marker rescue). This strategy placed the gene by homologous recombination into the J region of the vaccinia virus genome and therefore resulted in a TK^- virus (Mackett *et al.*, 1982) which was subsequently plaque purified 3 times in the TK^- 143 B cell line

under Bromo-deoxyuridine (BuDr) selection. Stocks were prepared in CV 1 cells as described in chapter 2. The presence of the gene was verified by both Southern blotting of the recombinant virus DNA (Fig. 8.1) and plaque hybridisation techniques (Fig 8.2), using a random primed, ^{32}P-labelled full-length mTGF β_2 gene as the probe.

N.B. Extra references relevant to the construction of recombinant vaccinia viruses are given in chapter 2.

Bioassay for TGF β_2.

143 B cells in EMEM plus 5 % heat-inactivated FCS were grown to confluency in Linbro 24 well plates and subsequently infected with the recombinant TGF β vaccinia virus or a non-TGF β control vaccinia virus (MOI = 5.0). At 1, 5, 17, 24, 48 and 72 hour intervals post-infection, supernatants were collected from the infected cells and UV-irradiated (5 minutes at approximately 10 cm) to inactivate the vaccinia virus. The supernatants from the control vaccinia virus infected cells were collected at the 72 hour time point only. Supernatants were also collected from 143 B cells that had been cultured over the same time period but not challenged with virus. These supernatants were then tested for their ability to inhibit the proliferation of the Mv-1-Lu cell line as determined by tritiated thymidine (1.0 μCi/well) incorporation. The method for this assay as well as the procedure for the determination of unknown TGF β values has been recently reviewed by Meager (1991). TGF β is often secreted *in vitro* as a latent form which requires physical or chemical treatment for activation. After testing the normal supernatants for TGF bio-activity, the assay was repeated with supernatants which had been heated to 100^{0}C for 3 minutes to activate any TGF present as the inactive precursor form (Lawrence *et al.*, 1985). Recombinant TGF β1 was used to calibrate the assay. It has been reported that TGF β1 and TGF β2 have equivalent inhibitory activity

in Mv-1-Lu cell bioassays, although this property is not universal for all cell types (Jennings *et al.*, 1988).

Growth of VV-TGF β_2-TK$^-$ in Swiss (athymic) Nude mice.

Female nude mice (6-8 weeks old) were infected with either VV-TGF β_2-TK$^-$ or a similarly constructed TK$^-$ control virus whose growth was not attenuated by the inserted gene (for this study, VV-IL 5-TK$^-$) at the doses of 5 x 10^6 pfu or 1 x 10^7 pfu intravenously (i.v). The mice were then observed for mortality as an indication to whether the presence of the TGF β gene was inducing *in vivo* enhanced or attenuated growth of the vaccinia virus.

Growth of VV-TGF β_2-TK$^-$ in thymic CBA/H mice.

Female CBA/H mice (6-8 weeks old) were infected i.v with 5 x 10^6 pfu of VV-TGF β_2-TK$^-$ or VV-IL 5-TK$^-$. At days 2, 4 and 7 post-infection the mice were sacrificed by cervical dislocation and the ovaries removed for virus growth titration. The protocol for vaccinia measurement in the ovaries was performed as described in the methods section of chapter 3.

Natural Killer (NK) cell activity in the spleens of VV-TGF β_2-TK$^-$ infected mice.

CBA/H mice (n = 3; 6-8 weeks old) were infected with 1 x 10^7 pfu VV-TGF β_2-TK$^-$ i.v. Control mice (n = 3) were infected with the same dose of VV-WR-TK$^-$. At day 2 post-infection the mice were sacrificed by cervical dislocation and the spleens collected into ice-cold RPMI plus 10 % heat-inactivated FCS. Spleens were also collected from non-infected mice for NK cell activity testing. The preparation of the spleen cells, labelling of YAC-1 target cells and the measurement of specific lysis were

all performed as described in the methods section to chapter 5.

Cytotoxic T lymphocyte (CTL) activity in the spleens of VV-TGF β_2-TK$^-$ infected mice.

CBA/H mice (n = 3; 6-8 weeks old) were infected with 1 x 10^7 pfu VV-TGF β_2-TK$^-$ i.v. Control mice (n = 3) were infected with the same dose of VV-WR-TK$^-$. Six days post-infection the mice were sacrificed by cervical dislocation and the spleens collected into ice-cold RPMI plus 5 % heat-inactivated FCS. Spleens were also collected from non-infected mice for CTL activity testing. The preparation of the spleen cells, labelling of L929 target cells and the measurement of specific lysis were all performed as described in the methods section to chapter 5. The L929 (target) cells were infected with VV-WR-TK$^-$ at a MOI of 20 during ^{51}Cr labelling.

* The experiments performed for this chapter were all done at least twice unless otherwise indicated. Please note that (n =) refers to the number of animals or samples in an experimental or control group.

RESULTS.

Expression of vaccinia virus encoded TGF β2 *in vitro*.

The time course of TGF β2 production in *in vitro* infected 143 B cells is described in Fig. 8.3. As can be seen there is a gradual increase in TGF β2 bioactivity with peak activity being reached at 24 hours post infection. TGF β2 bioactivity in supernatants collected at the 48 and 72 hours post infection time points were not significantly different compared to the 24 hour time point. The supernatants for the above described data had been heat-activated before the assay. Supernatants which did not have an activating step before the assay did display TGF β bioactivity, but it was generally not as pronounced or as consistent (ie, TGF β levels did not increase consistently with increased time) as that found for the heated samples (data not shown). This indicates that there is only partial activation of the TGF β secreted from VV-TGF β infected 143 B cells. Supernatants from 143 B cells infected with a control vaccinia virus at the same dose did not display any detectable inhibition of Mv-1-Lu cell proliferation (data not shown).

Growth of vaccinia virus encoding TGF β_2 (VV-TGF β_2-TK$^-$) in Swiss nude mice.

The growth of VV-TGF β_2-TK$^-$ in athymic Swiss nude mice was monitored as the relative survival of these mice compared to nude mice infected with a control vaccinia virus. Fig. 8.4 shows the kinetics of the survival in VV-TGF β_2-TK$^-$ versus VV-IL 5-TK$^-$ infected mice at 2 virus doses. At the 1×10^7 dose there was a slight, but statistically not significant increase in the survival of the VV-TGF β_2-TK$^-$ infected mice and this is reflected in the mean time to death (MTD) figures (Table 8.1). The 5×10^6 pfu dose showed a more pronounced increase in survival for VV-TGF β_2-TK$^-$ infected

mice compared to mice infected with the control virus. However, statistical analysis of the MTD for VV-TGF β_2-TK$^-$ infected mice again did not exhibit a significant difference when compared to the MTD for the control virus infected mice.

TABLE 8.1 The mean time to death (MTD) of Swiss nude mice infected i.v with either VV-TGF β_2-TK$^-$ or VV-IL 5-TK$^-$ (n = 5 for each group).

Virus dose (pfu/mouse)	**Mean Time to Death (days) ± SEM**	
	VV-IL 5-TK$^-$	**VV-TGF β_2-TK$^-$**
5 x 10^6	14.4 ± 0.6	18.6 ± 3.6
1 x 10^7	12.4 ± 0.2	13.8 ± 0.7

At both virus doses there was no significant difference in the MTD of mice infected with VV-TGF β_2-TK$^-$ compared to VV-IL 5-TK$^-$ ($0.29 > p > 0.10$).

Growth of VV-TGF β_2-TK$^-$ in the ovaries of thymic CBA/H mice.

Figure 8.5 describes the comparative growth of VV-TGF β to the growth of the control virus in the ovaries of mice at intervals of 2, 4 and 7 days post-infection (p.i.). At day 2 p.i. there was a decrease in the growth of the VV-TGF compared to the control virus but this was not significantly different. At both days 4 and 7 p.i. lower vaccinia virus titres were observed in the ovaries of VV-TGF infected mice but as with the day 2 time point there was not a statistically significant difference found when compared to the growth of the control virus.

Serum samples from both control virus and VV-TGF β infected mice had no

detectable TGF β bioactivity at the peak of virus growth (ie, day 4 p.i.; data not shown).

Natural Killer (NK) cell activity in CBA/H mice infected with VV-TGF β_2-TK$^-$.

Fig. 8.6 describes the comparative NK cell activity in CBA/H mice infected with VV-TGF β_2-TK$^-$ compared to mice infected with a control virus. The NK cell activity was not significantly different in VV-TGF β_2-TK$^-$ infected mice compared to mice similarly infected with the control vaccinia virus. At day 2 post infection in CBA/H mice the growth of VV-TGF β was not significantly different to that of the control virus (Fig. 8.5). This correlation to virus growth supports the finding that NK cell activity is not increased.

Cytotoxic T Lymphocyte (CTL) activity in CBA/H mice infected with VV-TGF β_2-TK$^-$.

Fig. 8.7 describes the activity of CTLs at day 6 p.i. in mice infected with VV-TGF β compared to mice infected with a control virus. There was not a significant difference in CTL activity for VV-TGF β infected mice compared to mice infected with the control virus. This supports the growth data (Fig. 8.5) which shows that at the time of peak CTL activity there was no difference in vaccinia virus growth in the ovaries of VV-TGF β infected versus control virus infected CBA/H mice. The lysis of non-infected targets did not exceed 5 % for either virus.

SUMMARY.

This chapter describes some characteristics of a recombinant vaccinia virus encoding TGF β2 (VV-TGF β). TGF β bioactivity could be detected in the supernatants of cells which had been infected with VV-TGF β, but to see consistent activity the supernatants had to be boiled to activate latent TGF β molecules expressed by the recombinant virus. Studies showed no differences in growth characteristics compared to a control virus for both athymic nude mice and thymic CBA/H mice. There were also no differences found for both splenic NK cell and CTL activities in VV-TGF β infected mice versus animals infected with a control virus. These results may reflect the *in vitro* findings, which suggest that TGF β is not fully activated under normal physiological conditions after expression from a vaccinia virus vector.

Fig. 8.1 Southern blots* of the viral DNA from, **1)** the parent vaccinia virus (VV-WR-L929), and **2)** the recombinant vaccinia virus encoding TGF β2 (VV-TGF β2-TK$^-$).

Fig. 8.2 Hybridisation* of viral plaques in 143 B cells from, **1)** the parent vaccinia virus (VV-WR-L929), and **2)** the recombinant vaccinia virus encoding TGF β2 (VV-TGF β2-TK$^-$).

*A random primed, ^{32}P-labelled full length mTGF β2 cDNA was used as the probe.

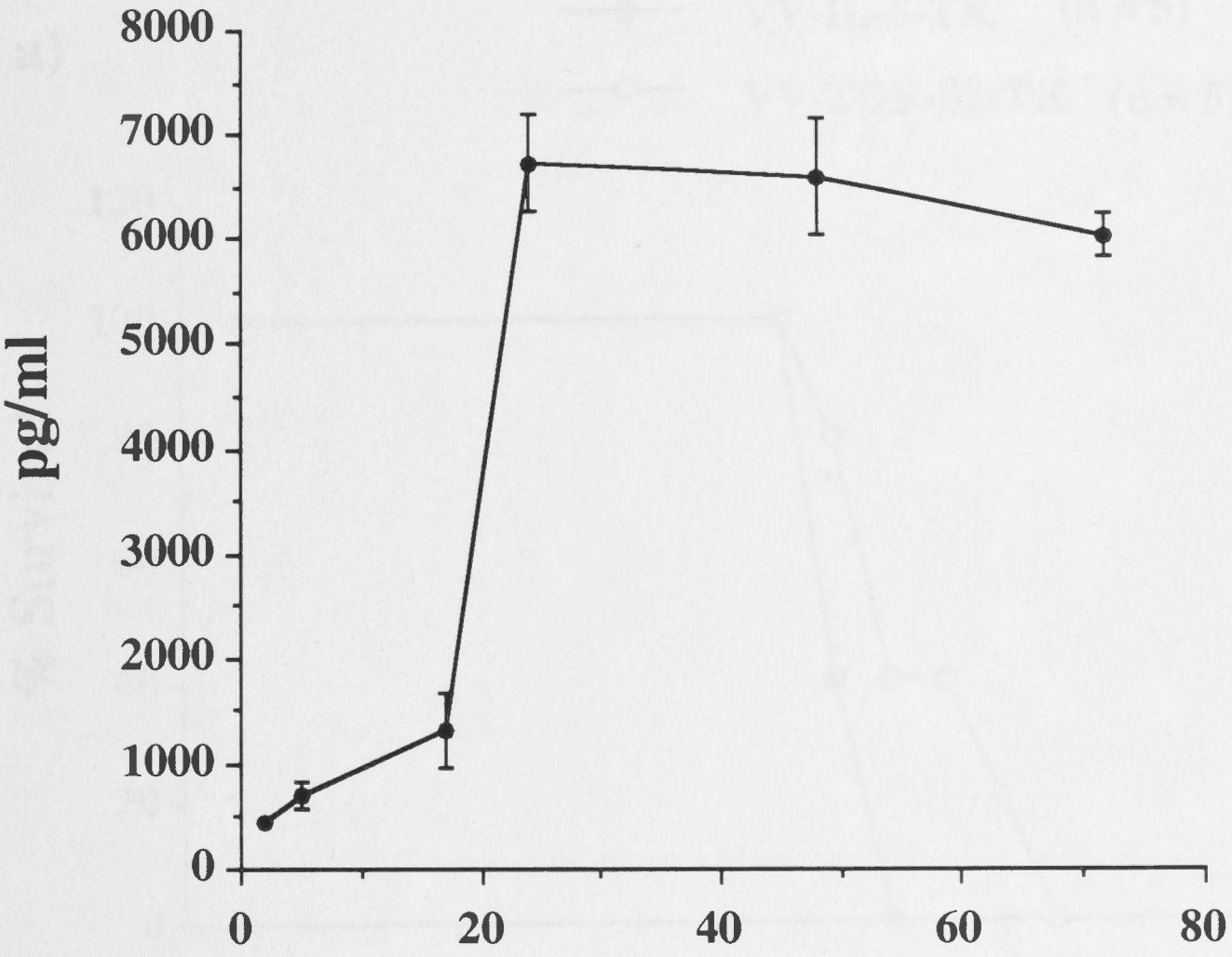


Figure 8.3 TGF β bioactivity in boiled supernatants from 143B cells infected with a recombinant vaccinia virus encoding TGF β2 (VV-TGF β2–TK$^-$).

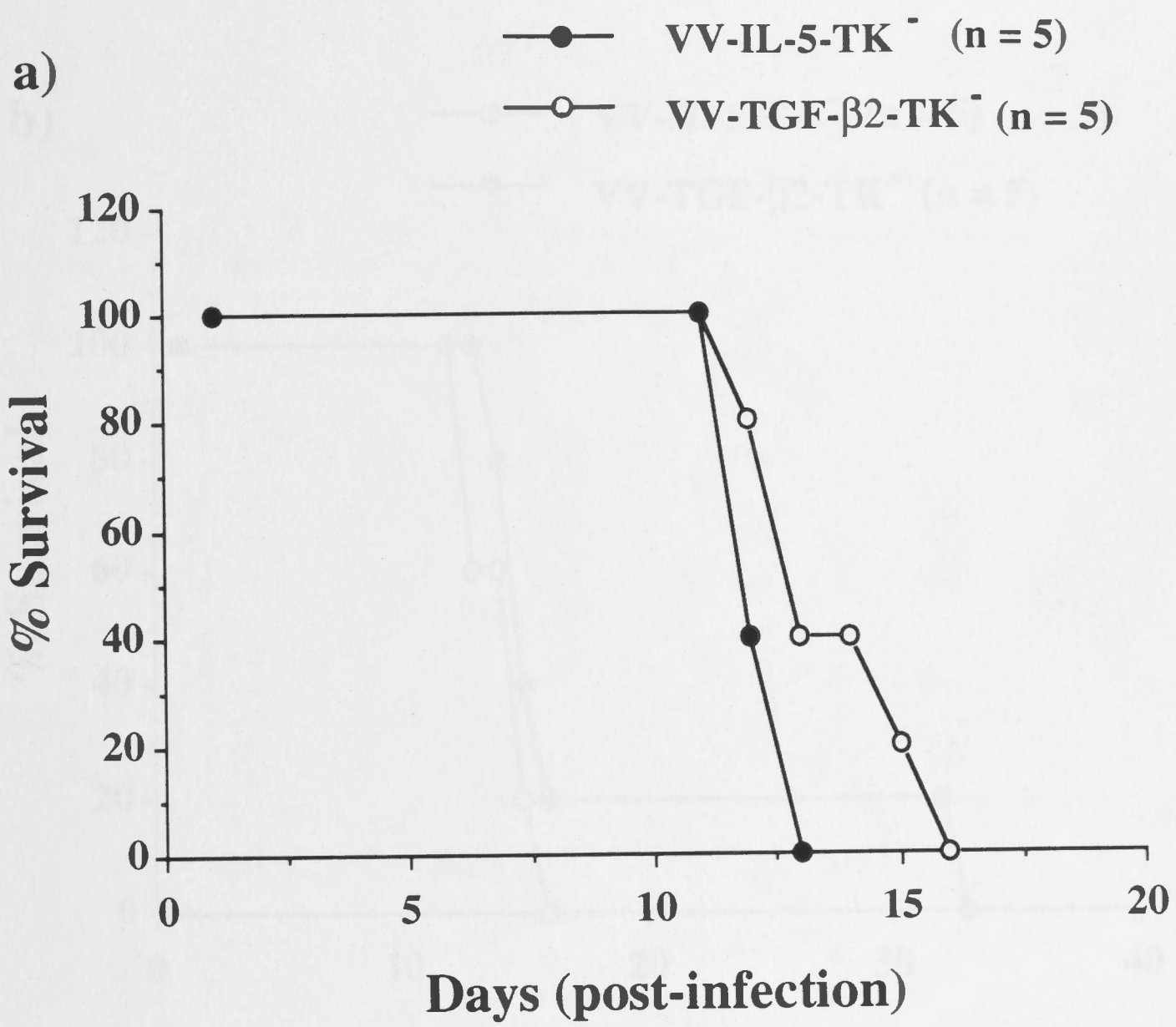


Figure 8.4 a) Survival of Swiss Nude mice infected with VV-TGF β2 versus a control vaccinia virus at the dose of 1×10^7 pfu (i.v).

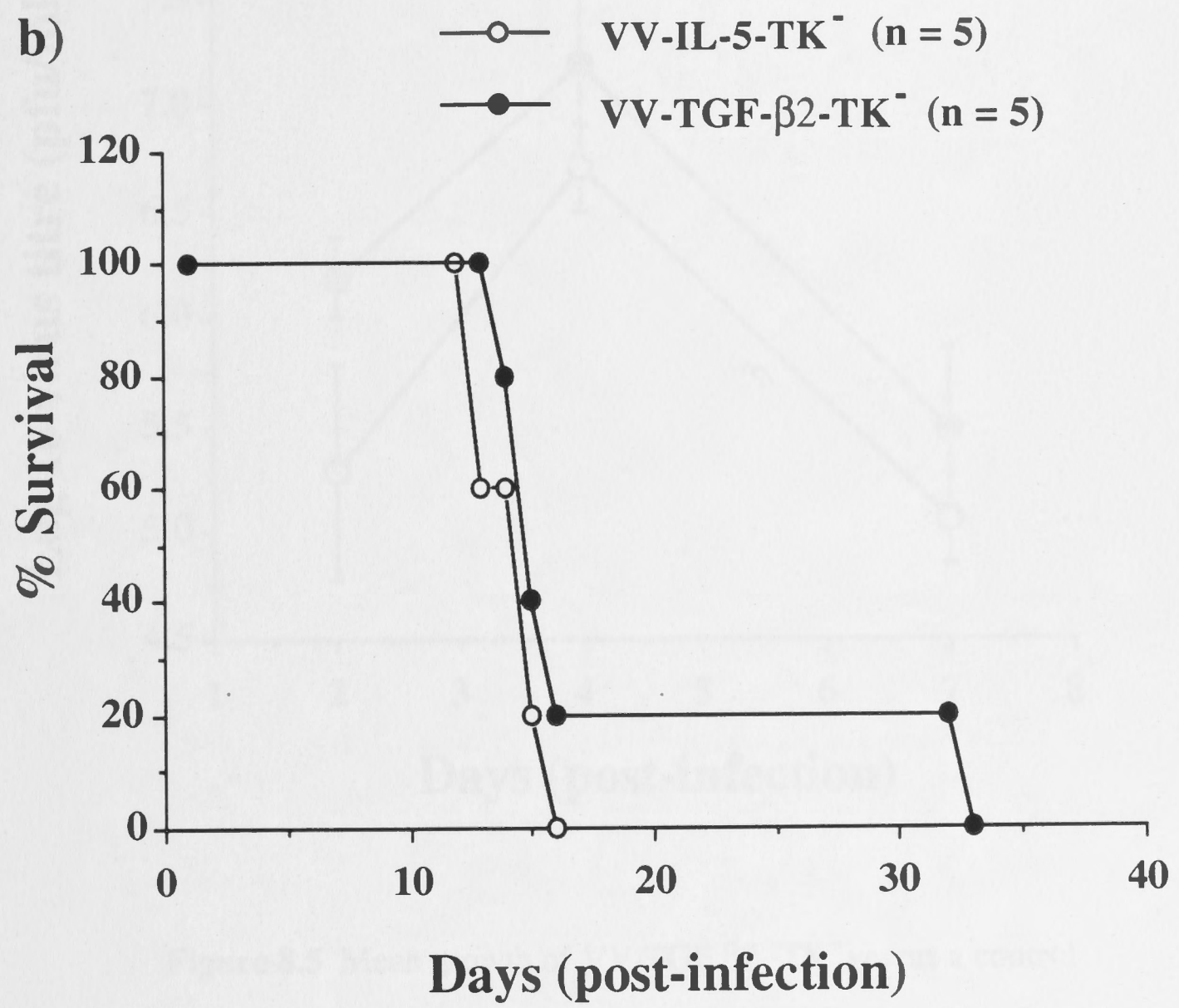


Figure 8.4 b) Survival of Swiss Nude mice infected with VV-TGF β2 versus a control vaccinia virus at the dose of 5×10^6 pfu (i.v).

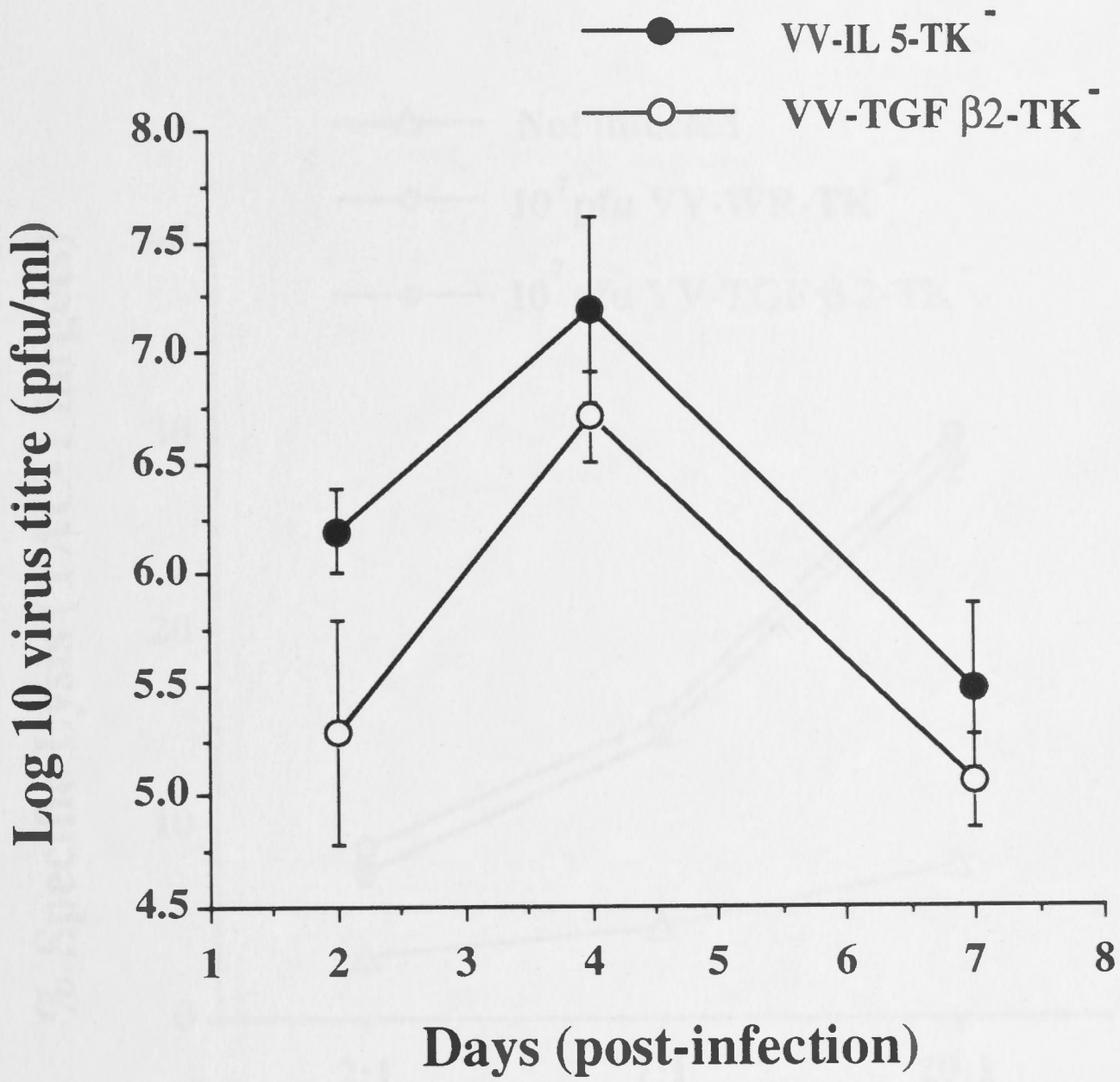


Figure 8.5 Mean growth of VV-TGF β2–TK⁻ versus a control Vaccinia virus in the ovaries of CBA/H mice at days 2, 4 and 7 post-infection.

There were no significant differences in virus titre between VV-TGF β2 and the control virus at day 2, 4 or 7 post-infection ($0.35 > p > 0.13$).

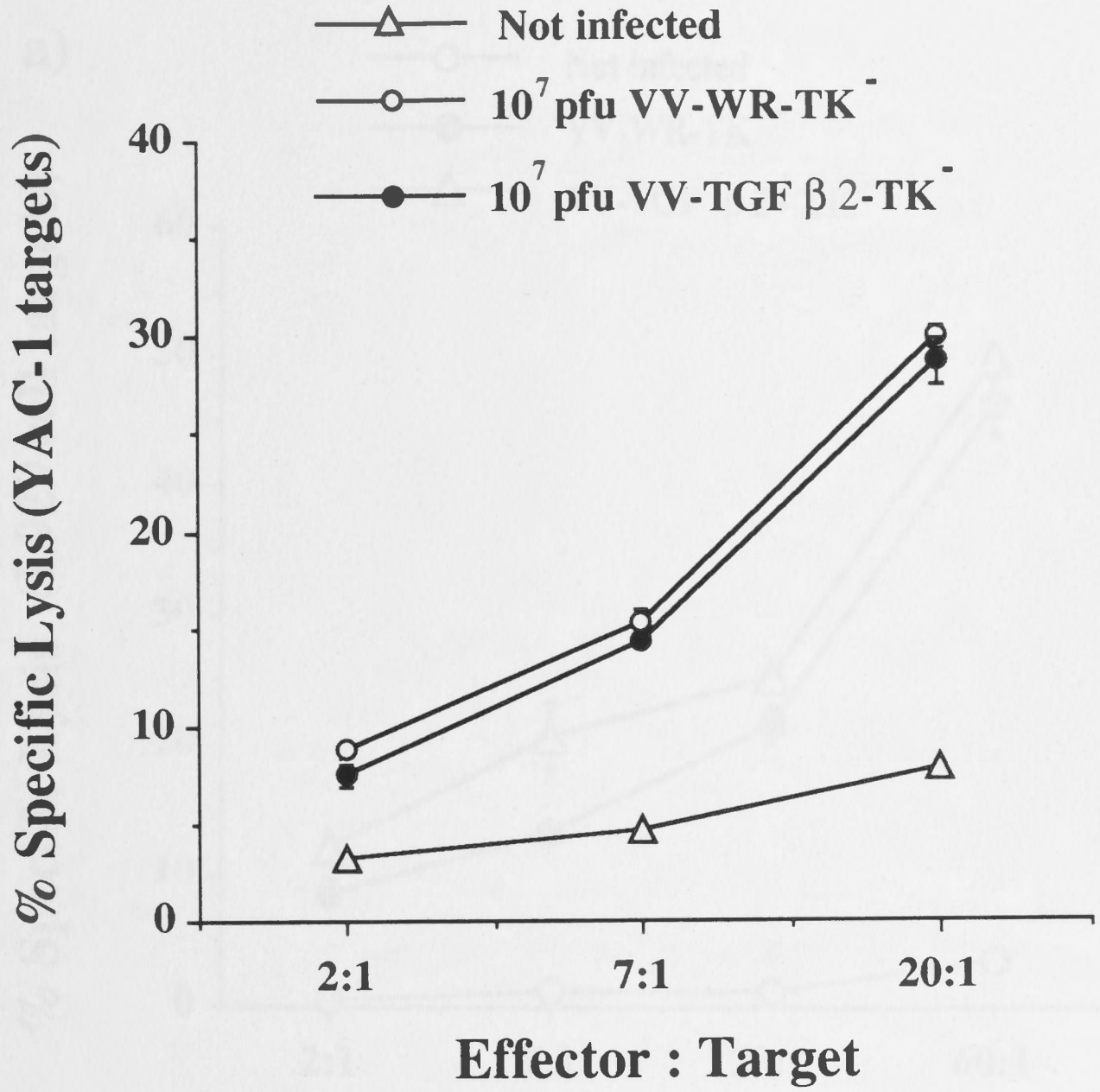


Figure 8.6 Natural Killer (NK) cell activity against YAC-1 target cells in the spleens of CBA/H mice infected with VV-TGF β2 versus a control virus.

*The above described data were the results of a single experiment.

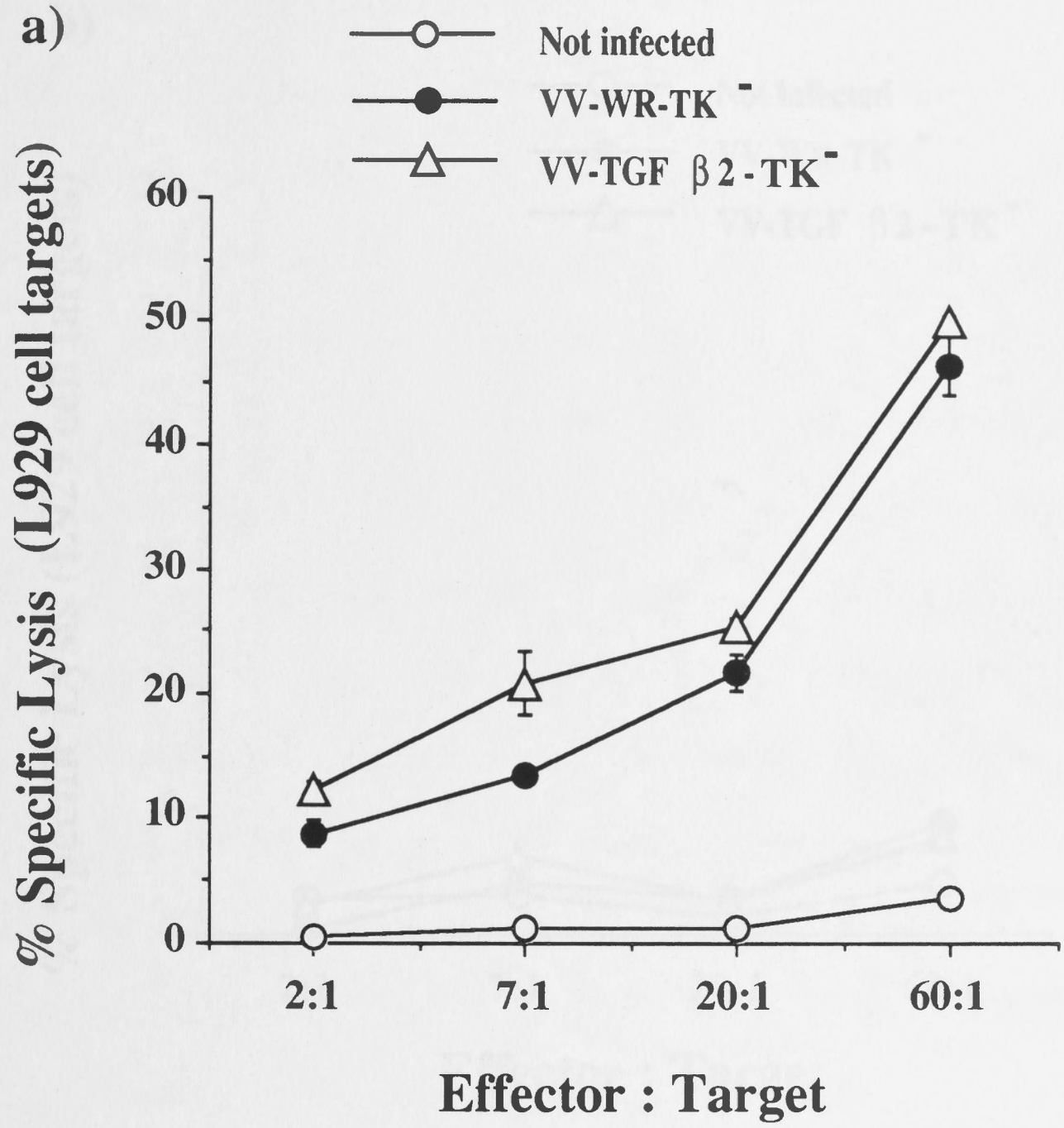


Figure 8.7 a) Cytotoxic T Lymphocyte (CTL) activity against Vaccinia virus-infected target cells (MOI = 20) from the spleens of VV-TGF β2 infected mice versus control virus infected mice.

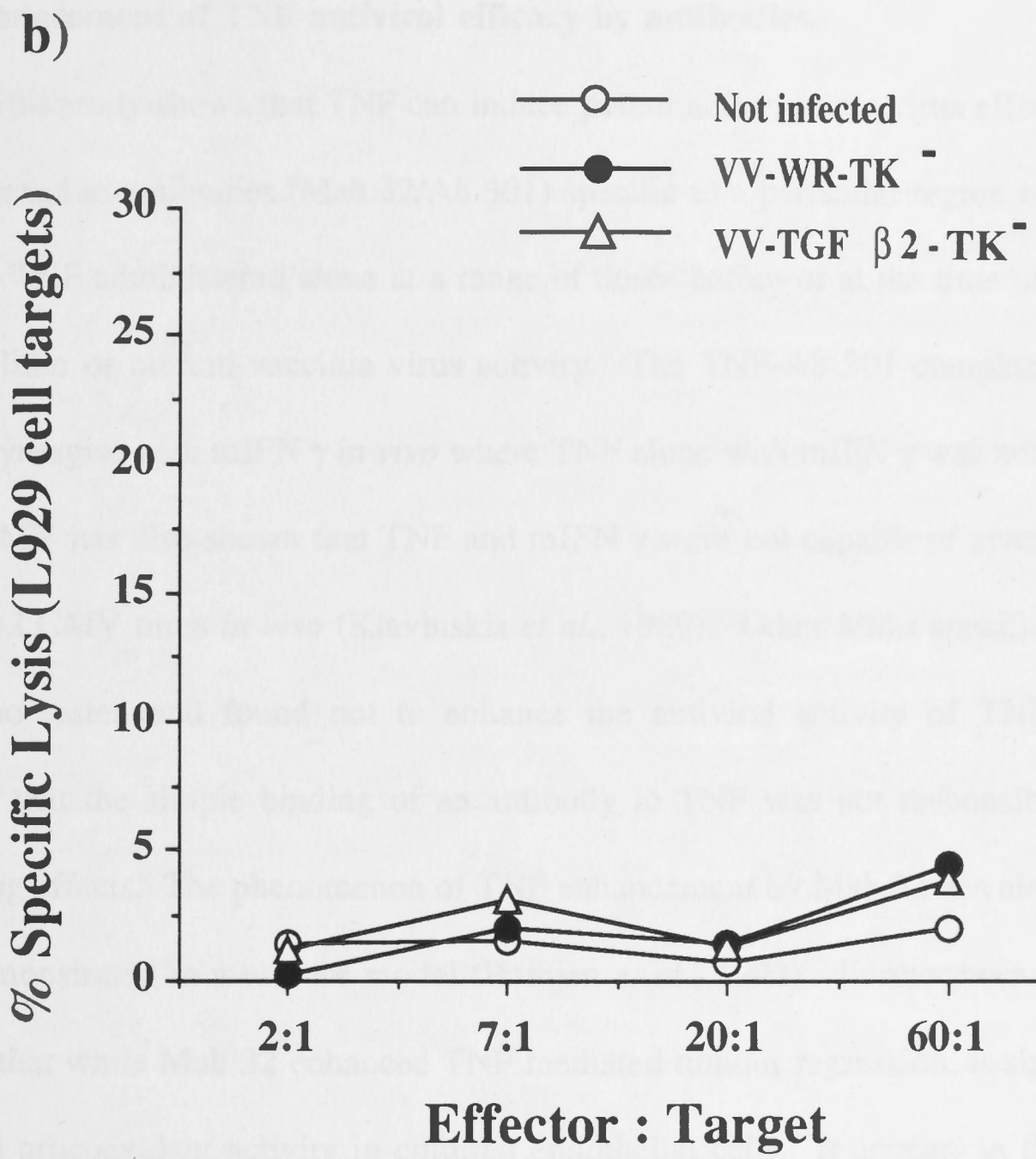


Figure 8.7 b) Cytotoxic T Lymphocyte (CTL) activity against non-infected target cells from the spleens of VV-TGF β2 infected mice versus control virus infected mice.

*The above described data were the results of a single experiment.

Chapter 9.

DISCUSSION and CONCLUSIONS.

9.1 Enhancement of TNF antiviral efficacy by antibodies.

This study shows that TNF can induce potent anti-vaccinia virus effects *in vivo* if complexed to antibodies (Mab 32/Ab 301) specific to a particular region of the TNF protein. TNF administered alone at a range of doses before or at the time of infection showed little or no anti-vaccinia virus activity. The TNF-Ab 301 complex was also able to synergise with mIFN γ *in vivo* where TNF alone with mIFN γ was not. A study with LCMV has also shown that TNF and mIFN γ were not capable of synergistically reducing LCMV titres *in vivo* (Klavinskis *et al.*, 1989). Other Mabs specific to hTNF were also tested and found not to enhance the antiviral activity of TNF, thereby showing that the simple binding of an antibody to TNF was not responsible for the enhancing effects. The phenomenon of TNF enhancement by Mab 32 has also recently been demonstrated in a tumour model (Rathjen *et al.*, 1992). Furthermore that study showed that while Mab 32 enhanced TNF mediated tumour regression, it significantly inhibited procoagulant activity in cultured endothelial cells. It appears in the tumour model that there can be selective enhancement of the biological activities of TNF with Mab 32. Antibody 301 has also been shown to enhance the anti-tumour efficacy of TNF (Rathjen & Aston, in press).

The treatment of mice with TNF in complex with univalent Fab' fragments of Mab 32 showed a weak antiviral effect. The reduction of virus titre observed with 0.5 mg Fab 32 plus TNF was not as pronounced as that found with the whole Mab 32 plus TNF. A possible explanation may be that the removal of the Fc region from the monoclonal antibody results in a more labile Fab' region (Rathjen, pers. comm.) and

therefore the longer more involved preparation procedures needed to produce the Fab' fragments could have partially compromised enhancing activities. A recent study has shown that bivalency and/or Fc mediated effects were not important in the ability of Mab 32 to augment the anti-tumour effects of TNF as Fab 32 enhanced TNF to the same degree as whole Mab 32 (Rathjen *et al.*, 1992).

The phenomenon of antibody enhancement has been known of for several years in relation to endocrine hormone activity. To decide on mechanisms of antibody enhancement of TNF it is therefore useful to consider the past literature concerning endocrine hormone enhancement by antibodies. Favoured mechanisms of endocrine hormone enhancement include, slow release (depot effect), bivalency and Fc region effects, conformational changes induced in the protein by the antibody, and receptor "restriction" effects (reviewed by Aston *et al.*, 1989). The evidence provided above in the studies with Fab' fragments suggest that the induction of conformational changes in the TNF molecule due to Mab/Ab binding could be very likely. It also appears quite likely that the antibodies may be acting in a similar fashion to carrier proteins that afford protection to other proteins, like TNF, from *in vivo* degradation, and hence extend physiological half-lives. A recent report has shown that bioactive oligomeric TNF will convert into an inactive monomeric form at picomolar concentrations under physiological conditions and the authors conclude that this instability might be important in the regulation of TNF cytotoxicity (Corti *et al.*, 1992). This finding raises interesting questions regarding the ability of Mab 32 or Ab 301 to prevent the conversion of TNF into inactive monomers and hence introduce greater stability to the TNF molecule. Change in conformational structure could also contribute to prolongation of TNF half-life. Results in chapter 5 show that TNF in complex with Ab 301 did persist longer in the peritoneal cavity of CBA/H mice compared to mice

injected with TNF alone, therefore supporting this notion. It is most likely that the underlying mechanisms of antibody enhancement are multi-faceted. Another putative mechanism which might be related to antibody-induced conformational changes is the Mab 32 mediated enhancement of TNF binding to cells *in vitro*. This will be discussed in more detail later.

A concept that is gaining more acceptance is that different regions of the TNF molecule are responsible for its various biological activities. The TNF epitopes to which Mab 32 bind have been described (Rathjen & Aston, in press), and it could be that the antibody is selectively inhibiting certain TNF functions by blocking the regions responsible for those activities. The evidence discussed earlier (Rathjen *et al.*, 1992) certainly supports this concept. Some very recent work (Van Ostade *et al.*, 1993) has shown that mutant forms of the TNF molecule have altered or restricted biological activities. Through this approach, ie., altering particular regions of TNF, it was found that it was possible to stop the binding of TNF to one class of receptor (ie, TNF-R75; this is discussed in more detail later), and thereby influence the biological activity. It was found that one mutant retained its antitumour activity in Nude mice, and like the Rathjen *et al.*, (1992) studies concluded that such approaches to TNF therapy could be of great use as the desirable (eg, antitumour) effects could be retained while the undesirable side-effects are lost.

9.1.1. Response of Herpes Simplex Virus type 1 (HSV-1) growth *in vivo* to TNF treatment with and without Ab 301.

The response of HSV-1 in CBA/H mice to the TNF therapies, with or without Ab 301, contrasted dramatically with that found for vaccinia virus infection. TNF treatments alone induced significant reductions in virus growth both in the ovaries and

the adrenal glands. Of foremost interest, however, were the observations that at TNF doses of 1.0, 2.0 or 6.0 μg there was no enhancement of anti-HSV-1 effects by Ab 301 *in vivo*. Both of these findings are in stark opposition to what was found with vaccinia virus infection in CBA/H mice. To see any enhancement of TNF's antiviral activity against HSV-1 the TNF dose had to be reduced to a level which alone had no effect on HSV-1 growth (this enhancement was not statistically significant). There is evidence that vaccinia is intrinsically resistant to TNF and IFN γ. For example a study has shown that while the translation of HSV proteins was blocked by the combined action of TNF with low doses of IFN γ, vaccinia virus translation continues unaffected in cells treated with both of these compounds (Feduchi *et al.*, 1989). These results suggest that *in vivo* TNF's antiviral capability cannot be augmented further if the virus, for example HSV-1, is already very sensitive to this antiviral cytokine. The fact that Mab 32 and Ab 301 do augment the *in vivo* antiviral effects of TNF against a normally TNF/IFN resistant virus such as vaccinia virus raises the possibility that TNF/Ab 301 or Mab 32 complexes induce additional antiviral mechanism(s). This could be, by virtue of the ability of Ab 301/Mab 32 to stabilise TNF *in vivo*, that there is interference with an event which occurs after translation. The fact that HSV is vulnerable to very early TNF/IFN mediated antiviral effects (ie, inhibition of HSV protein translation; Feduchi *et al.*, 1989) may make redundant the presence of an enhancing antibody with TNF as secondary non-specific and early TNF-induced effects would not be required. In addition, the dichotomy in the response of vaccinia virus and HSV-1 to *in vivo* TNF therapy with or without enhancing antibodies is underpinned by interesting *in vitro* findings concerning possible direct antiviral effector mechanisms. This will be discussed in greater detail later (section 9.5).

9.2 Host immunity to Vaccinia virus and HSV-1 infection.

For both vaccinia virus and HSV-1 the mouse strains BALB/c and C57/B6 were tested for their response to various TNF therapies. It has previously been shown that resistance to herpes simplex virus infection in inbred mouse strains is variable and genetically controlled. For example, BALB/c mice have been shown to be very susceptible to HSV infection whereas C57/B6 mice are resistant to infection with HSV (Zawatzky *et al.*, 1981). Similar observations have been made in studies with ectromelia (mousepox) virus (Buller & Palumbo, 1991). It was found that C57/B6 mice were better able to control the growth of vaccinia virus compared to either BALB/c or CBA/H mice. In the present study, in regards to vaccinia virus, different responses to exogenous antiviral agents between these three inbred mouse strains were observed, for example BALB/c mice were not responsive to either TNF plus Ab 301 or mIFN-γ alone whereas the CBA/H and C57/B6 strains were. The variation in response to TNF and IFN γ either alone or together between the 3 mouse strains was more pronounced for HSV-1 infection. This was particularly seen when BALB/c mice were compared to C57/B6 mice. The BALB/c mice showed no significant decrease in viral growth in response to the exogenous cytokine therapies whereas dramatic reductions in HSV-1 growth were observed in B6 mice, particularly with the TNF alone treatment. It therefore appears that responses to cytokines in the context of viral infection has a genetic basis.

Pox virus infection elicits a range of immune responses both of a non-specific and specific nature. Non-specific factors like interferon have been shown to protect mice from ectromelia infection (Imanishi *et al.*, 1980; Imanishi *et al.*, 1981) and complement activation is thought to be involved (eg, increased phagocytosis via C3b receptors; Hirsch, 1982) although there has not been a direct demonstration of its role.

Natural killer (NK) cells have been found to be elevated in mice inoculated with vaccinia virus (Welsh *et al.*, 1979; Stitz *et al.*, 1985) and there is evidence that mouse strains resistant to ectromelia have higher NK cell activity than susceptible strains (Jacoby *et al.*, 1989). Recent work on a recombinant vaccinia virus expressing IL 2 has shown that elevated NK cell activity and corresponding increases in IFN γ production are responsible for the attenuated growth of this virus (Karupiah *et al.*, 1990a; Karupiah *et al.*, 1990b). NK cells are obviously important in the early response to pox virus infection but evidence showing the crucial role of T cells (Hirsch *et al.*, 1968; Blanden, 1970; Blanden, 1971) later in infection indicates that the NK cell and other early non-specific responses do not control the virus. Of the T cell response which occurs later in pox virus infection in mice (day 6 to day 7 post infection) there is a significant cytotoxic ($CD8^+$) T cell component which appears to be responsible for *in vivo* protection as increased CTL activity correlates with viral clearance (Hapel & Gardner, 1974; Blanden & Gardner, 1976). In light of the evidence showing that CTL activity is largely responsible for clearance of virus, it is therefore not surprising that antibody is not important for the clearance of a primary pox virus infection, although specific antibody is apparently important in preventing reinfection (Buller & Palumbo, 1991).

In HSV infections, it is generally agreed that all arms of the immune response are at work in the acute phase of primary infection by this virus (Wildy & Gell, 1985). Some early interest in immune responses to HSV focused on differences on resistant and susceptible mouse strains (Zawatzky *et al.*, 1981; Engler *et al.*, 1981). These studies showed no differences in the production of macrophage migration inhibitory factor and antibodies between resistant and susceptible mouse strains. A greater production of HSV-induced interferon (IFN) was detected in the resistant mouse strain and this appeared to correlate with increased NK activity. Further work showed very

convincingly the necessity of IFN α and IFN γ production as primary factors in the resistance of mice, particularly C57/B6 mice, to HSV infection (Zawatzky *et al.*, 1982; Stanton *et al.*, 1987). Recent work on the role of T cells in HSV infection have demonstrated that in humans $CD4^+$, MHC class II restricted T cells dominate the CTL response whereas in the mouse $CD8^+$, MHC class I CTLs are predominant with $CD4^+$ cells being a minor component of this response. It has been demonstrated that their ($CD4^+$ cells) role in the regulation of the CTL responses may be greater than their numbers indicate (Kolaitis *et al.*, 1990). This point is supported by the findings of Jennings *et al.*, (1991) who showed that $CD4^+$ T lymphocytes were important for the generation of the primary $CD8^+$ CTL response to HSV in C57/B6 mice. $CD4^+$ T lymphocytes were, however, not found to be of importance to the secondary $CD8^+$ CTL response.

As discussed above IFN appears to be a crucial factor in the resistance of mice to HSV infection. Other cytokines have also been reported as effective in controlling HSV growth. These cytokines include TNF (Rossol-Voth *et al.*, 1991), IL 3 (Chan *et al.*, 1990), IL 1α, IL 2 and M-CSF 1 (Berkowitz & Becker, 1992). The work described in this thesis also shows TNF to be capable of inhibiting the *in vivo* growth of HSV-1. The evidence suggests that HSV is sensitive to a range of cytokines, the early endogenous action of these factors can, in some cases, significantly lower the viral load. This alone, however, cannot control the proliferation of the virus in a normal physiological sense, as evidenced by the upregulation of T cell activity later in infection. What could be exciting, however, is that the administration of certain exogenous cytokines displays potent anti-HSV capabilities in a primary infection, although, in the mouse model this potency appears to rely on the genetic background of the host.

Studies on the host immune response to vaccinia virus in CBA/H mice primed with TNF plus enhancing antibody (Ab 301) showed that the enhanced antiviral effect of TNF plus Ab 301 (with and without IFN γ) was not sensitive to gamma-irradiation. Gamma irradiation neutralises proliferative immune cell responses, but not macrophage activity (which is of great relevance to future discussion in this chapter). In further studies it was discovered that NK cell and CTL responses were reduced in animals that had been treated with TNF plus Ab 301 before infection in comparison to animals treated with TNF alone. In the NK cell study this reduction in activity was even more pronounced if mIFN γ had been co-administered with the TNF-Ab 301 complex. For both CTL and NK cell studies there was no difference in activities between mock (ie, PBS) treated and TNF alone treated mice, which supports the earlier findings that TNF alone has almost no effect on the response to vaccinia virus *in vivo*. The NK cell result particularly indicates that the TNF-antibody therapy is inducing or augmenting potent antiviral activities earlier than day 2 post infection. Results displayed in table 3.8 support this, as vaccinia virus growth in TNF plus antibody treated mice was considerably lower than in control treated mice at day 1 post infection. These results are supported by the findings of Sambhi *et al.*, (1991) who showed that a recombinant vaccinia virus encoding the gene for murine TNF α had its growth significantly attenuated compared to a control recombinant vaccinia virus. The attenuation of the growth of the VV-TNF was also noticeable as early as day 1 post infection and this reduction of growth was found not to be due to the enhancement of either cellular or antibody responses. The authors concluded that the attenuation of growth seen with VV-TNF must be due to the direct antiviral effect of TNF at the foci of infection.

At day 1 post-vaccinia virus infection no outstanding differences were noted in the profile of the resident peritoneal cell population nor was there any evidence of

increased endogenous IFN activity in the peritoneal washings of mice which had been treated with TNF plus Ab 301 24 hours before infection. Also in day 1 post infection peritoneal washings no increase was found in reactive nitrogen intermediate (RNI) levels in TNF plus Ab 301 pretreated mice. This finding supports past observations on the effects of TNF on macrophages. Ding *et al.*, (1988) found that resident murine peritoneal macrophages could not be primed with TNF to produce RNI species upon PMA stimulation. TNF could however prime for the increased production of reactive oxygen intermediates (ROIs) in this model. IFN γ could prime resident macrophages for RNI as well as ROI production and further to this synergised with TNF to produce RNI levels 6 times greater than that induced by IFN γ alone. These findings could be an attractive explanation for the earlier observations of TNF plus Ab 301 antiviral effects and the synergy of this complex with mIFN γ to further reduce the *in vivo* growth of vaccinia. Like the Ding *et al.* model the cytokines were present with macrophages (and other peritoneal cells) for a period before stimulation with vaccinia virus. If both nitrogen and oxygen radicals were present, as could be envisaged with the co-administration of TNF plus Ab 301 and IFN γ, additional reactive species such as peroxynitrite molecules (Beckman *et al.*, 1990) would be induced which could contribute more potency to the inflammatory reaction on viral infection. The induction of inflammatory responses involving oxygen/nitrogen radicals is a very early and non-specific mechanism for the restriction of pathogen growth and could be an important factor in explaining the early restriction of virus growth seen after TNF and/or IFN γ treatment. A more detailed discussion on the possible antiviral action of free-radicals will be presented later in this chapter.

9.3 The influence of enhancing antibodies on TNF, TNF-receptor interactions.

An interesting property of Mab 32 and to a lesser extent Ab 301 was their ability to enhance the binding of TNF to cells *in vitro*. The cell lines tested were L929 (mouse) and HeLa (human). Both of these cell lines also displayed enhanced internalisation of TNF if Mab 32 was present. This enhanced binding and internalisation correlated with the enhancement of TNF antiviral effects in L929 cells, but not in HeLa cells. This raises the notion that while TNF receptors are ubiquitous and virtually all cell types tested will bind TNF (Jaattela, 1991) post-receptor binding effects depend on the cell type and different intracellular mechanisms. TNF signal transduction has been recently reviewed by Kronke *et al.*, (1990). Receptor binding and internalisation has also been found not to be the significant correlating factor in TNF-induced *in vitro* cytotoxicity (Kull *et al.*, 1985; Yoshie *et al.*, 1986; Tsujimoto *et al.* 1985). As TNF is a pleiotropic protein it seems likely that the presence of TNF receptors on cell lines resistant to TNF cytotoxicity are associated with one or more of the other activities of TNF.

Recent attention has re-focussed on the TNF receptor. It has been discovered that there are 2 immunologically distinct murine TNF receptors of approximately 55 kD [TNF-R1] or 75 kD [TNF-R2] (Hohmann *et al.*, 1989; Engelmann *et al.*, 1990a; Brockhaus *et al.*, 1990) and now there is evidence implicating each of these classes of receptor in different biological functions. The crux of recent investigations on the individual receptors was the finding that TNF-R2 will bind murine TNF with high affinity but does not bind human TNF at all. TNF-R1 will bind mouse and human TNF with equal affinity. It was therefore suggested that human TNF activity in mouse tissues or mouse cell lines would be mediated through TNF-R1 and mouse specific activities of TNF mediated via TNF-R2 (Lewis *et al.*, 1991). A further study (Tartaglia,

et al., 1991) exploited this specificity of murine TNF for TNF-R2 as well as using as agonists polyclonal antisera highly specific for the extracellular domain of either TNF-R1 or TNF-R2. The use of these specific antibodies particularly allowed for the elucidation of biological activities associated with each receptor. Using this approach it was found that the induction of proliferation by TNF on mouse thymocytes and the murine cytotoxic T cell line CT-6 mapped to TNF-R2 only. TNF activities mediated via TNF-R1 in the cell lines tested were cytotoxicity and the induction of the protective enzyme manganese SOD (MnSOD). TNF has been shown previously to induce MnSOD in a wide range of cell lines as well as various mouse organs (Wong & Goeddel, 1988b). The above findings demonstrate that in the enhancement of human TNF antiviral effects *in vitro* by Mab 32 in L929 cells only TNF-R1 is important. Scatchard analysis of the ^{125}I-hTNF alone binding suggested that only one class of receptor was being bound in both HeLa cells and L929 cells, although in the presence of Mab 32 ^{125}I-hTNF binding kinetics for both cell lines were representative of the presence of a second low-affinity class of receptor as well. How this enhanced binding occurs is again open to speculation. Mechanisms discussed earlier in relation to enhancement of endocrine hormones by antibodies are again relevant. Whatever the mechanism in L929 cells there is definitely an antiviral pathway linked to TNF-R1 and the presence of Mab 32 with the hTNF enhances this antiviral signal. It is most likely that the intracellular pathway responsible for the antiviral effects is very closely related to the cytotoxic pathway which emanates from the same receptor.

With the presence of antibodies in the TNF enhancement model, receptor cross-linking may be important in the generation of an amplified antiviral signal. Work by Engelmann *et al.*, (1990b) showed that antibodies to a soluble form of the TNF receptor had TNF-like activity. It was concluded from this study that TNF itself is not directly

involved in intracellular signalling, but rather it is the binding events which initiate the stimulation of events such as receptor clustering, which enables more productive TNF-receptor cross-linking, and hence effective intracellular signalling. The importance of cross-linking events was inferred by the observations that univalent fragments of the TNF-receptor antibodies had no TNF-like activity and this could be restored by cross-linking anti-immunoglobulin antibodies. At this point it is useful to note that for TNF to be biologically active it must exist in its trimeric form. As a trimer TNF can therefore bind to more than one receptor.

9.3.1 The effect of TNF and/or IFN γ pretreatment on normal and HSV-1-infected cell TNF receptor expression.

Further to the above proposed mechanism of enhanced TNF binding with Mab 32, results in chapter 6 (Fig. 6.5 a) also show that exogenous hTNF can induce increased expression of TNF receptors in a dose dependent manner. The presence of Mab 32 with TNF results in a non significant enhancement of TNF receptor expression. This indicates that TNF with and without Mab 32 can increase proliferation in fibroblasts resulting in a higher *de novo* production of cellular proteins such as TNF-R. There is evidence showing that in inflammatory responses cells may shed TNF receptors (Van Zee *et al.*, 1992). It appears however that over periods of time, even in the presence of TNF, cells can regenerate receptors for TNF. This also suggests that autocrine activity by TNF in certain physiological situations might be essential to TNF receptor replacement. L929 cells which had been treated with TNF at various doses plus or minus Mab 32 and infected 24 hours later with HSV-1 were also examined for their TNF receptor expression. The major finding here was that in non-TNF treated cells HSV-1 infection itself induced a significant increase in the expression of TNF

receptors. This effect of HSV-1 saturated any differences due to TNF treatment alone. The upregulation of TNF receptor expression by HSV-1 infection is very interesting particularly since this virus is sensitive to TNF both *in vitro* and *in vivo*.

Of interest also was the effect of IFN γ alone and with TNF in infected and uninfected L929 cells. This interest stems from the early results showing an *in vivo* antiviral synergy when IFN γ was co-administered with TNF plus Ab 301. There has been conjecture in the past that the modulation of TNF receptors by IFN γ is important to the TNF:IFN synergy. It has been found that IFN γ enhances the specific binding of TNF to receptors in a range of cell lines, without significantly effecting receptor binding affinity (Aggarwal *et al.*, 1985; Ruggiero *et al.*, 1986; Tsujimoto *et al.*, 1986). The increased synthesis of TNF receptors has been found to be the mechanism associated with the IFN γ induced enhancement of TNF:receptor binding (Tsujimoto & Vilcek, 1986). Results shown in figure 6.6a. support this finding since IFN γ induced the expression of TNF receptors. The presence of TNF alone with IFN did not significantly effect this, but there were slight increases in receptor expression if the L929 cells were treated with IFN and 100 or 200 ng TNF plus Mab 32. On infection of these cells with HSV-1 24 hours after cytokine treatment the profile of TNF receptor expression changed. The most dramatic finding was that in IFN pretreated cells HSV-1 infection resulted in the expression of significantly fewer TNF receptors in comparison to both mock-infected cells treated with IFN alone and cells only infected with HSV-1. The presence of TNF alone with IFN showed slight TNF-R increases on HSV-1 infection with the doses of 100 ng and 200 ng TNF being more effective with Mab 32 and the 10 ng TNF dose not being significantly influenced by Mab 32. Vaccinia virus was not tested in this model as it has been found that pox viruses contain regions in their genome which are homologous to TNF receptor (Upton *et al.*, 1991; Howard *et*

al., 1991). Therefore there is a possibility that the TNF receptor expression detected in this situation would be of viral and not cellular origin. To our knowledge HSV does not contain TNF receptor homologs.

These results show that *in vitro* IFN γ can influence the expression of TNF receptors both in non-infected and HSV-1 infected L929 cells. The relative importance of IFN γ to the observed antiviral synergy with TNF plus Ab 301 is still open to speculation. From the results in chapter 3 (Table 3.5), however, it does appear that for the best antiviral effect, IFN γ should be administered either before TNF plus Ab 301 or at the same time as this complex. This effect may underlie a more significant role for IFN γ in TNF receptor modulation in antiviral responses. It has been found previously that for the LPS/IFN γ-induced production of antimicrobial nitric oxide by murine macrophages either LPS and IFN had to be administered simultaneously or exposure to IFN was required before LPS. If LPS was given before IFN, little response was seen (Lorsbach & Russell, 1992). In the area of TNF tumour cytotoxicity it is felt that IFN upregulation of TNF receptors is a contributing factor, although, probably not a major one (Tsujimoto *et al.*, 1986). Intracellular events post receptor binding and internalisation are almost certainly of equal if not greater importance to the biological manifestations of IFN γ and TNF plus antibody interactions.

9.4 The direct *in vitro* enhancement of TNF antiviral effect by Mab 32 and the involvement of a TNF-induced cytotoxic factor.

The antiviral effects of TNF can be directly enhanced by Mab 32 *in vitro* for both vaccinia and HSV-1. Together with the evidence showing that the *in vivo* antiviral effects of TNF plus Ab 301 occur as early as day 1 post-infection it appears that the TNF-induced mechanism responsible for the control of *in vivo* virus growth is an early-

acting and non-specific event which occurs directly in the infected tissues. This has been alluded to earlier in the discussion on the role of the host immune response to virus infection. Further investigation of the direct *in vitro* antiviral response uncovered an interesting and possibly important mechanism, particularly in regard to HSV-1 infection. Cells that had been exposed to TNF and infected 24 hours later with a low, normally non-cytopathic dose of HSV-1 displayed significant CPE compared to those not treated with TNF which showed little or no CPE after infection with the same dose of virus. This enhanced CPE effect was most dramatic and severe if the L929 cells had been exposed to TNF plus Mab 32 before HSV-1 infection. This effect was definitely virus-induced as TNF alone and TNF plus Mab 32 treated, mock-infected controls showed no CPE. This enhanced CPE furthermore correlates with the reduced growth of HSV-1 in TNF treated cells.

The dose of virus used would only initially infect 1 in 10 cells (MOI = 0.1), therefore it can be hypothesised that a factor is being produced at the foci of HSV-1 infection which induces severe CPE in the surrounding TNF pre-exposed cells thereby restricting the growth of the virus. Further investigation of this phenomenon demonstrated that it was unlikely that a secreted protein factor was responsible. Also, enhanced degradation of cellular DNA was not important to this effect which indicates something about the nature of the agent involved in the enhanced CPE. At the 48 hour time point the DNA extracted from these cells was largely intact even though significant CPE could be observed in the relevant treatments. This could indicate that an external factor is operating in this enhanced CPE which potently damages cellular membranes before significant nucleic acid damage is observed. The indication therefore is that the physical damage to the cells is due to an agent which does not induce damage in the target cells via a protein-induced intracellular pathway, but acts from outside of the cell.

Free radical involvement in the enhanced CPE was discovered through studies with the antioxidant butylated hydroxyanisole (BHA). The addition of BHA after HSV-1 infection had 2 effects in TNF plus Mab 32 pretreated cells; 1) the considerable CPE was reversed and 2) the growth of HSV-1 increased dramatically. In cells which were pretreated with PBS (ie, not exposed to TNF) and exposed to BHA a massive increase in HSV-1 growth was also seen but there was also CPE, which was not seen in non-BHA, PBS-treated cells. So in regard to CPE there is an opposite effect between cells exposed to TNF before infection and those that were not. The growth of HSV-1 in the PBS treated cells exposed to BHA was approximately 1.0-1.5 logs greater than in cells pretreated with TNF and exposed to BHA. It may be that the CPE seen in the PBS treated cells is a result of the massive growth of the virus in the cells independent of free radical damage, or the dose of BHA used was not sufficient to scavenge all the free radicals produced due to this enhanced HSV-1 growth.

It has been shown that BHA can reverse TNF cytotoxicity in L929 cells and WEHI-164 cells (Schulze-Osthoff *et al.*, 1992; Brekke *et al.*, 1992). It is important to note that the inclusion of BHA with TNF (ie, before infection) showed no effect on HSV-1 growth when compared to non-BHA treated cultures. This indicates that there was not significant TNF-induced, free radical mediated L929 cell killing before infection. This also confirms the previous comment that the factor inhibited by BHA is virus-induced. Also pre-exposure of L929 cells to BHA alone had no effect on HSV-1 growth compared to non-BHA exposed controls.

BHA, in addition to its antioxidant properties, can inhibit arachidonic acid pathways. To test whether arachidonic acid metabolism was important to the BHA-induced effects, cyclooxygenase/lipoxygenase inhibitors were tested in L929 cells infected with HSV-1. None of the 3 inhibitors tested induced an increase in HSV-1

growth *in vitro*. The role of reactive nitrogen intermediates (RNIs) was also considered. Through studies with a nitric oxide synthetase (NOS) inhibitor, N^G-ethyl-L-arginine acetate (NELA), it was found that RNIs were not important to the *in vitro* growth of HSV-1 both in TNF-treated and non-TNF treated cells. It therefore appears most likely that free oxygen radicals are responsible for the BHA-induced effects. It appears from the experimental evidence that the role of free oxygen radicals in this model is 2 fold. Firstly, regardless of whether the cells had been pre-exposed to TNF or not, free oxygen radical production is a major component of the direct antiviral response to HSV-1. Neutralising free oxygen radicals results in dramatic increases of HSV-1 growth. Secondly, prior exposure of cells to TNF sensitises those cells to the cytopathic effects of free oxygen radicals and this appears to be a significant factor in the further restriction of HSV-1 growth by TNF.

Although RNIs were not important in the *in vitro* growth of HSV-1 it cannot be ruled out as an important effector molecule *in vivo*, particularly in association with cytokines. IFN γ and TNF have been found to induce an "L-arginine-dependent cytotoxic effector mechanism in murine macrophages" which turned out to be nitrite and nitrate (Drapier *et al.*, 1988). There have been several examples in the literature of IFN/TNF-induced RNIs being responsible for increased killing of parasites by macrophages (Munoz-Fernandez *et al.*, 1992; Li *et al.*, 1992; Nacy *et al.*, 1991). The possible role of TNF, lymphotoxin and IL 1 in the induction of RNIs in a malaria model have recently been examined also (Rockett *et al.*, 1992). The evidence concerning parasite infection seems very convincing in that cytokine induction of RNIs is an important early effector system. Specific evidence on virus infection to my knowledge is not yet available. There will be further discussion on this topic later in the chapter regarding the role(s) of down-regulatory cytokines such as TGF β.

9.4.1 Free oxygen radicals.

The *in vivo* role of oxygen radicals in virus-infection has been considered in the past. Studies have highlighted the contribution of oxygen radicals to the pathogenicity of influenza virus infection of the mouse lung and maintained that direct free radical related cytotoxicity was one aspect of the overall pathology (Akaike *et al.*, 1990; Maeda & Akaike, 1991). Whether oxygen radical production was also important to virus-growth restriction was not made clear. The role of reactive oxygen intermediates (ROI) has recently been discussed in HIV infection (Muller, 1992) and this review raised another aspect of radicals and virus infection. As well as the possible impairment of macrophage and neutrophil ROI production by HIV, it also appears that ROIs can activate the intracellular transcription factor NF-κB which stimulates HIV replication. The full implications of such findings are as yet unknown.

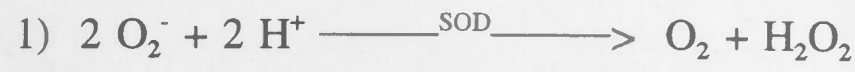
There is evidence which supports the relationship of free radical systems and antiviral cytokine activity. For example among the range of IFN-inducible proteins two, xanthine oxidase and 2,3-dioxygenase, are involved in oxygen metabolism (Samuel, 1987). It has also been discovered that both IFNs and TNF are capable of inducing superoxide generation (Lappegard *et al.*, 1988; Larrick & Wright, 1990). This relationship has also been investigated directly in antiviral models. Pottathil *et al.* (1981) discovered that by inhibition of SOD activity with the copper chelating agent, diethyldithiocarbamate (DDC) they reduced the antiviral (VSV) effectiveness of exogenous IFN. This was found in a variety of mouse cell lines, including L929 cells, and one human cell line. In a more recent study HeLa cells were stably transfected with a SOD gene which resulted in CuZn SOD activities ranging from 1.6-7.3 times the normal level (Huang *et al.*, 1992). These cells were treated with different concentrations of human IFN α and later challenged with VSV. The response to this

treatment varied according to the amount of SOD expressed. Cells producing up to a 3-fold increase displayed an inverse relationship between CuZn SOD activity and induction of an IFN-mediated antiviral state, namely, the higher the CuZn SOD activity the lower the sensitivity to IFNs antiviral effect (ie, more IFN was needed to produce an antiviral state). Clones with SOD activity between 4.0-7.3 times higher showed a direct relationship between sensitivity to IFN and SOD activity (ie., the higher SOD activities required less IFN to induce an antiviral state). These results are in partial conflict with the Pottathil *et al.* study although it should be noted that the model system and cell lines used were different. Both studies taken together however do highlight the role of superoxide generation and SOD activity in the modulation of the IFN-induced antiviral state. It does appear however that SOD is pivotal in determining whether the IFN antiviral activity is augmented or diminished. The activity of SOD appears not to simply neutralise free oxygen radical production by inhibiting the superoxide radical, but could encourage the production of more deleterious radical species such as the hydroxyl radical and this appears from the latest evidence of Huang *et al.* to depend in part on the degree of SOD activity present at the site of virus infection.

There has been a recent examination of the role of SOD and free radical production in ischaemia-reperfusion injury (Mao *et al.*, 1993). At higher concentrations SOD can enhance injury, and this study found that in such cases the toxicity of free SOD could be attributed to hydroxyl radicals resulting from an increased Fenton-type reaction. These findings support the argument that SOD activity can result in the potentiation of free radical damage.

The contribution of free radicals and antioxidants in human disease has been recently reviewed (Halliwell *et al.*, 1992) and provides insights into how free radical

chemistry can apply to biological systems. A point of great relevance to the above discussion concerns how from the superoxide radical (O_2^-) which alone under normal physiological conditions is not very toxic, more toxic radical species can eventuate via Fenton reaction chemistry. To summarise the following equations will be used;



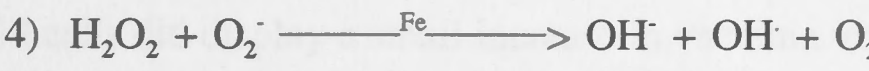
1) $2\ O_2^- + 2\ H^+ \xrightarrow{SOD} O_2 + H_2O_2$

2) $H_2O_2 \xrightarrow{Catalase} O_2 + H_2O$

OR

3) $Fe\ (II) + H_2O_2 \longrightarrow OH^- + OH^{\cdot} + Fe\ (III)$

Which can be summarised by the following equation;



4) $H_2O_2 + O_2^- \xrightarrow{Fe} OH^- + OH^{\cdot} + O_2$

As described above the product of SOD catalysis of the superoxide radical leads to hydrogen peroxide (1) which in the presence of the enzyme catalase is broken down into harmless by-products. However, in the presence of transition metals like iron, Fenton reactions can occur which ultimately produce toxic hydroxyl radicals which can have deleterious biological effects such as lipid peroxidation, nucleic acid oxidation, intracellular calcium fluctuation among other things. As can be seen in (4) superoxide can play a role in the generation of hydroxyl radicals which lends the more accurate term Superoxide-Driven Fenton reaction. Therefore, although alone this molecule may be harmless, in this situation it can have toxic consequences and this is largely due to superoxide's ability to reduce certain ferric chelates. Of course in mammalian systems

iron is normally bound into proteins such as haemoglobin, transferrin and ferritin, thereby limiting free iron availability (Halliwell *et al.*, 1992). However in pathological conditions where tissue damage occurs it might be that iron and other transition metals could be released at the site of injury thereby supplying the catalyst required for such free radical-mediated activity.

9.5 Vaccinia virus infection behaves differently to BHA treatment compared to HSV-1 infection.

It appears from the data that free radical production is not important to the direct *in vitro* restriction of vaccinia virus growth in L929 cells whether with or without TNF pretreatment. BHA-reversible CPE was observed in L929 cells which had been TNF plus Mab 32 pretreated and vaccinia virus infected but this CPE was not as intense as had been observed in similarly treated cells infected with HSV-1. In addition, TNF plus Mab 32 pretreated L929 cells did display a small increase in vaccinia virus growth after BHA exposure but in general there was no dramatic effect on vaccinia growth in TNF pretreated, BHA exposed L929 cells. This was in contrast to the massive increase of growth in HSV-1 infected cells after BHA exposure. In L929 cells treated with PBS prior to vaccinia virus infection this contrast (with what was found for HSV-1) was more pronounced. There was no visible CPE in PBS treated/vaccinia infected cells with BHA exposure and the growth of vaccinia in these cells was almost identical to that found for similarly treated cells not exposed to BHA after infection. (With HSV-1 infection CPE was observed in the L929 cells with BHA treatment). These results indicate that in L929 cells which have not been exposed to TNF before infection, free radical production in infected cells is not an important mechanism in the restriction of vaccinia virus growth which is in bold opposition to HSV-1. To highlight this

difference further studies on the comparative production of SOD in HSV-1 versus vaccinia infected L929 cells showed greater SOD activity in HSV-1 infected cells especially if the cells had been TNF plus Mab 32 pretreated. The predominant form of SOD produced in both HSV-1 and vaccinia virus infected L929 cells was CuZn SOD (cytosolic SOD) which supports the earlier cited work (Pottathil *et al.*, 1981; Huang *et al.*, 1992) that showed the involvement of the same SOD species in their virus-infection models. Mitochondrial SOD (Manganese SOD) has been shown by Northern analysis to be induced in a variety of cells directly by TNF and it is speculated that this SOD species is important in the regulation of direct TNF cytotoxicity (Wong & Goeddel, 1988b). The biochemical assay system used in the present study is probably not sensitive enough to pick up MnSOD activity. No SOD activity was detected in PBS, TNF or TNF plus Mab 32 treated and mock-infected L929 cells. This difference does emphasise, however, the apparently unique role of CuZn SOD in virus infected cells. Of prime importance in the above discussed work is the apparent lack of free radical involvement in the inhibition of vaccinia virus infection. In comparison, HSV-1 infection of the same cell line displayed a very strong free radical component. This suggests therefore that the reason(s) for this difference is not of cellular, but of viral origin. The ability of viruses, particularly vaccinia virus, to encode for proteins of immunological relevance has recently become an area of considerable interest.

The circumventing of certain aspects host immune response by virus is not restricted only to free radical systems as alluded to above. Earlier (pages 113-114) the finding that pox viruses can encode proteins with significant homology to cellular TNF receptors was discussed. The authors of the work on myxoma virus (Upton *et al.*, 1991) concluded that this viral TNF-R homolog could be an important secreted "virulence factor". More recently it has been discovered that myxoma virus also

encodes a homolog of the IFN γ receptor which as a protein can bind IFN γ and inhibit its activity (Upton *et al.*, 1992). It has also been recently found that vaccinia virus encodes a secretory glycoprotein that functions as a soluble IL 1 receptor. The authors of this work found that this IL 1 receptor homolog had a role in modulating the severity of the virus-induced disease, particularly the systemic acute phase responses (Alcami & Smith, 1992). An example of virus-encoded immune virulence factors has also been found in the herpes virus family. Epstein-Barr virus (EBV) has been found to contain a gene very similar to IL 10 (Moore *et al.*, 1990). This molecule is thought to play a significant part in the virulence of this virus. As well as down-regulating Th1-type immune function (eg, TNF and IFN γ production) it also stimulates the proliferation of B cells which is the predominant cell type that EBV infects. It was thought that HSV-1 could bind and penetrate vascular cells via a fibroblast growth factor (FGF) receptor; this was later shown to be possibly due to a HSV-1 associated FGF-like protein (Kaner *et al.*, 1990; Baird *et al.*, 1990). However, it has recently been shown by other investigative procedures that the FGF receptor is not required for HSV-1 infection (Mirda *et al.*, 1992) therefore contradicting the earlier reports.

Examples of the resistance of various viruses to interferon antiviral activity exist and adds more credence to the ability of viruses, through a range of mechanisms, to avoid immune detection and/or effect. For example there have been reports of resistance to the IFN-induced 2-5A oligosynthetase antiviral mechanism. Studies by Chebath *et al.* (1987) showed that Chinese hamster ovary (CHO) cells transfected with the 40 kD form of 2-5A oligosynthetase were resistant to infection by mengovirus but not VSV or HSV-2. Other work showed that similarly 2-5A transfected cells were resistant to encephalomyocarditis virus (EMCV) infection (Rysiecki *et al.*, 1989; Coccia *et al.*, 1990). Incidentally, both mengovirus and EMCV are picornaviruses. Another

study has shown that the resistance of vaccinia virus to interferons may be related to virally-induced modifications of the 2-5A synthetase antiviral pathway, for example by the vaccinia-mediated inhibition of the activation of the 2-5A dependent RNase (Paez & Esteban, 1987). It has also been discovered that another IFN-induced antiviral pathway, namely the P1/eIF-2 protein kinase pathway, can be modified by virus infection. Both DNA and RNA viruses are capable of this. There are various ways that viruses can modify this mechanism, for example, adenovirus and HIV can induce the production of RNA which blocks activation, whereas reoviruses and vaccinia can produce a protein which binds the activator RNA needed to initiate this process (Samuel, 1991). It therefore appears from the literature that there is overwhelming evidence showing that viruses can actively circumvent antiviral mechanisms. The work presented in this thesis provides evidence that in addition to the above discussed factors, viruses like vaccinia are capable of avoiding or neutralising free radicals in early antiviral responses.

9.6 Transforming growth factor-beta (TGF β) in vaccinia virus infection.

To study the *in vivo* effects of TGF β on vaccinia virus growth a recombinant vaccinia virus was made which encoded this protein. This approach has been used previously to study a range of cytokines and their effects on vaccinia virus (Ramshaw *et al.*, 1992). The preliminary evidence presented in this thesis showed that TGF β does not have a significant effect on the growth of vaccinia virus in either athymic nude mice or thymic CBA/H mice. It can therefore be concluded that TGF β is not a directly antiviral cytokine. The effect on CTL responses by TGF β in this model was also examined and it was found that no significant change was induced in animals infected with VV-TGF compared to a control virus. The comparative NK cell activities

between VV-TGF β and control infected mice were also found not to be significantly different. The NK cell result particularly is in contrast to the results of an earlier study using LCMV infection as a model (Su *et al.*, 1991). It was observed initially that TGF β was produced at a time that co-incided with the decline of NK cell activity. Closer examination revealed that NK cell proliferation was very sensitive to inhibition by TGF β both *in vitro* and *in vivo*. In contrast to this T cell proliferation was unaffected and it was hence suggested that TGF β might be important to the co-ordination of NK cell and T cell responses during virus infection. Another study has found however that TGF β actually inhibits CTL activity against LCMV (Fontana *et al.*, 1989) which again is in contrast to what was found with VV-TGF β. A possible problem in these studies could be related to the TGF β sub-type, because while the different types of TGF β share many biological properties, there are still differences. For example, it has been found that while TGF β1 expression was high in the spleen, TGF β2 and β3 expression was not detected in this organ (Barnard *et al.*, 1990).

TGF β has been shown to be secreted *in vitro* as a latent molecule. This property is viewed as an important mechanism in the regulation of TGF β as in this form TGF is unable to bind its receptor which therefore results in the loss of biological activity (Lawrence *et al.*, 1985; Roberts & Sporn, 1988; Barnard *et al.*, 1990). The results obtained with VV-TGF β suggest that there might be a problem with the *in vivo* activation of the TGF protein after virus-induced expression. To detect vaccinia virus-expressed TGF β in a bioassay (Fig. 8.3) heat treatment of the infected cell supernatants was required to see consistent and full TGF bioactivity. Plasmin has been identified as a possible *in vivo* activator of TGF β (Lyons *et al.*, 1990), but whether this protease is activated during a vaccinia virus infection is not known. It has been shown that TGF β in serum is also latent. This has been attributed to TGF forming a complex with α_2-

macroglobulin (α_2-M) which acts as the TGF serum carrier protein and confers to TGF all the properties described for latent TGF β (O'Connor-McCourt & Wakefield, 1987; Roberts & Sporn, 1988). So as well as TGF β being secreted as a latent form, for *in vivo* studies there is also the problem of TGF complexing to α_2-M. This may not affect the bioactivity of TGF that had been expressed via a recombinant vaccinia virus as the production of such recombinant cytokines are normally focused to specific sites of infection. To properly study the role of TGF β in recombinant vaccinia virus infection the latency problems discussed above need to be overcome. Possible approaches include the co-expression of TGF β with plasmin, or the construction of a recombinant vaccinia virus containing the TGF β1 gene which by site-directed mutagenesis has been manipulated so that it is expressed as the biologically active molecule (Brunner *et al.*, 1989).

The past literature on TGF β shows that its antiviral activities are ambiguous. A report by Poli *et al.* (1991) showed that TGF β potently inhibited the production of HIV in a chronically infected promonocytic cell line, and this was not due to IFN α induction. The HIV suppression by TGF β was also found in primary macrophage cultures infected *in vitro*, but not in either a chronically infected T cell line or primary T cell blasts. Another report has appeared which demonstrated that the monocytotropic HIV-1 isolate (ADA) had its infection parameters enhanced by TGF β. This enhanced effect on HIV production occurs in cells with an established infection or in cells with active virus production (Lazdins *et al.*, 1991). Further to this it was shown that TGF β was capable overcoming the lymphocytotropic restriction seen in many HIV isolates. The presence of TGF with HIV isolates that normally do not grow, due to various viral characteristics, in particular macrophages or T lymphocytes resulted in infection indicators similar to that found for HIV isolates with a tropism for monocytes (Lazdins

et al., 1992). Therefore, inhibition of HIV infectivity in mononuclear blood cells by TGF β is still controversial.

The role of TGF β has also been studied in terms of parasite infections. In this context TGF β is quite often discussed along with IL 4 and IL 10 by virtue of these 3 cytokines having the common ability to down-regulate immune responses to parasite infection (reviewed by Sher *et al.*, 1992). Silva *et al.* (1991) showed that in spleen cells, from *Trypanosoma cruzi*-infected mice, elevated levels of biologically active TGF β could be detected. On further investigation it was found that TGF β also blocked the IFN γ-induced restriction of parasite growth in mouse peritoneal macrophages and human macrophages. Treatment of mice with TGF β exacerbated parasitaemia and resultant mortality and decreased resistance to *Trypanosoma cruzi* in certain mouse strains. Similar findings were made for *Leishmania*, which is of considerable interest since the exclusive host cell for this parasite is the macrophage (Barral-Netto *et al.*, 1992). *Schistosoma mansoni* is another parasite which infects macrophages (at the schistosomula stage). Activation of murine peritoneal macrophages with IFN γ will kill the schistosomula *in vitro* via an L-arginine dependent mechanism involving RNI production. The cytokines IL 4, IL 10 and TGF β all inhibit this IFN-induced extracellular killing of schistosomula, and this correlates with the down-regulation of RNI production in these activated macrophages (Oswald *et al.*, 1992a). It has been found subsequently in regard to *Schistosoma mansoni* that the pathway responsible for the down-regulation of antimicrobial effect is different for IL 10 in comparison to IL 4 and TGF β (Oswald *et al.*, 1992b). The above evidence concerning parasite infection shows very clearly the crucial role of the macrophage in the early control of infection and most importantly the very significant part TGF β, and other cytokines, play in down-regulation of macrophage antimicrobial efficacy and associated enhanced disease.

In light of this information, plus the earlier discussed ambiguity associated with HIV infection and the down-regulation of NK cell responses to LCMV, it would appear that TGF β is not a good candidate as an antiviral cytokine. As well as these factors it has recently been discovered via the development of TGF β gene-targeted mice that TGF β suppresses systemic inflammatory responses (Kulkarni *et al.*, 1993). As concluded earlier, free radicals, which are important in inflammatory reactions, are potentially of great significance in the restriction of virus infection and TGF β may inhibit their production. Of additional interest is the possibility that TGF exerts subtle effects on other immune cell populations. For example, it has been mooted that TGF β has a significant role in the generation of immunological memory by possibly inducing the growth of committed memory cells (Swain *et al.*, 1991).

9.7 Future research.

As mentioned above there may be subtle effects seen on further study of VV-TGF β. A second recombinant TGF β virus with a co-expressed influenza haemagglutinin gene would prove very useful in the study of processes such as immunological memory and antibody isotype switching.

With the main thrust of the work in this thesis, namely TNF in viral infection, of particular importance is the more comprehensive study of early TNF-induced antiviral events. Work presented in this thesis has provided evidence for a contribution by free radicals to the early restriction of virus growth. However, this work was done in a continuous cell line. It would hence be of great use to study the observed *in vitro* effects of free radicals with and without TNF in the context of primary ovary cell cultures. Further to this, ultimate interest lies with the study of free radicals in virus control *in vivo*. It is possible to treat mice with substances like BHA and/or to

specifically block RNI synthesis with nitric oxide synthetase inhibitors. Although RNI production does not seem to be important in terms of a fibroblast cell line it is certain to have an important role in a heterogeneous *in vivo* cell population which includes macrophages. Earlier cited literature supports this position. Through the availability of recombinant vaccinia virus encoding cytokines like IFN γ and TNF it will also be possible to examine specifically the contribution of such cytokines to the induction of free radical systems against vaccinia virus infection *in vivo*. As yet the mechanisms of TNF and IFN direct antiviral effects *in vivo*, in the context of recombinant vaccinia, have not been fully elucidated and early free radical induction could be a major candidate. Regarding the other important issue of the down-regulation of immune responses to viruses, the availability of recombinant IL 4 and IL 10 vaccinia viruses, along with the recombinant TGF β vaccinia virus described in this thesis, could also provide unique opportunities for the examination of such cytokines in the subversion of early non-specific antiviral mechanisms like free radical production.

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